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Begin the article with a short summary.

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Submit an original and three copies of the manuscript, typed double spaced. Each page should begin with a new paragraph. Use accepted spelling, abbreviation, and hyphenation found in *Webster's New International Dictionary*. Footnotes should be typed at the bottom of the page on which they appear.

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Submit each table on a separate page, typed double spaced. Please use standard abbreviations; otherwise spell out.

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The Secretary of Health, Education, and Welfare has determined that the publication of this periodical is necessary in the transaction of the public business required by law of this Department. Use of funds for printing this periodical has been approved by the Director of the Office of Management and Budget through January 31, 1980.

Pharmacokinetic Rationale for Peritoneal Drug Administration in the Treatment of Ovarian Cancer [1]

Robert L. Dedrick,[2,*] Charles E. Myers,[3] Peter M. Bungay,[2] and Vincent T. DeVita, Jr [3,4]

SUMMARY

Evidence from the peritoneal dialysis literature suggests that the peritoneal permeability of a number of hydrophilic anticancer drugs may be considerably less than plasma clearance. Pharmacokinetic calculations indicate that such drugs administered ip in large volumes are expected to maintain a significantly greater concentration in the peritoneal space than in the plasma. This concentration difference offers a potentially exploitable biochemical advantage in the treatment of patients with presumed microscopic residual ovarian cancer confined to the peritoneal cavity.

[Cancer Treat Rep 62:1–11, 1978]

Ovarian cancer is the fourth leading cause of cancer death in women. In spite of the pronounced tendency for this tumor to remain localized to the peritoneum, even when far advanced, traditional forms of local treatment such as surgery and radiation therapy have failed to cure the majority of patients. While systemic chemotherapy has proved effective in inducing complete remissions in as high as 35% of patients with stage III or IV disease, true cures at this stage occur in < 10% of patients. Thus, it appears that for most patients the best that conventional therapy has been able to accomplish for both localized tumors treated with surgery and/or radiotherapy, and advanced tumors treated with drugs, is the reduction of bulky, clinically apparent tumors to a state where only microscopic disease persists. We would like to propose peritoneal dialysis with drugs known to be effective in ovarian cancer as a logical form of therapy in this setting.

Considerable information has accumulated on the factors which govern movement of chemicals across the peritoneal membrane. From this work, it appears that the peritoneal clearance of certain neoplastic agents is much lower than the plasma clearance. Because of this, ip administration of many drugs may be expected to result in a significant concentration difference between the peritoneal cavity and plasma. This concentration difference offers a potentially exploitable biochemical advantage. This paper discusses the pharmacokinetic parameters which would govern such local therapy and what conditions would be likely to optimize its effectiveness.

SIMPLIFIED ANALYSIS

The general kinetic features of ip drug administration are illustrated in figure 1. In this figure, the body is represented by a single compartment with volume of distribution (V_D) and drug concentration (C_p). The body communicates with fluid in the peritoneal cavity with volume (V) and concentration (C). The drug crosses the peritoneal membrane of area (A) by a diffusive process characterized by a permeability (P). The drug may also be carried by convective transport with transperitoneal fluid movement or lymph flow. The quantitative role of the convective terms is not well understood. It would appear that lymph flow is small and that other fluid shifts can be minimized by use of dialysate that is iso-osmotic with plasma. As commonly practiced in physiology, the intrinsic P is not measured separately but only in combination with the associated A.

The permeability-area product (PA) has the form

[1] Received July 20, 1977; accepted Oct 25, 1977.

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[3] Division of Cancer Treatment, National Cancer Institute, Bethesda, Md.

[4] We wish to thank Dr. Robert Popovich for helpful advice and Linda Gair for assistance in preparation of the manuscript.

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of a clearance. Its value is expressed in ml/minute, and the magnitude may be usefully compared with blood flows and clearance by various organs in assessing the relative quantitative significance of concurrent kinetic processes (21).

Mass balance equations may be written for each compartment in figure 1. If the volumes of distribution are constant, these take the form

$$V_D \frac{dC_p}{dt} = PA[C - RC_p] - kC_p \quad [1]$$

$$V \frac{dC}{dt} = PA[RC_p - C] \quad [2]$$

where k is the whole-body clearance by all processes and R represents the equilibrium dialysate to plasma C ratio (required to account for plasma protein binding).

Equation [1] must be modified somewhat if significant metabolism occurs in the liver because this would produce an effect similar to the first-pass phenomenon following oral drug administration. Apparently, most of the drug absorbed from the peritoneum enters the portal system (1); therefore, "bioavailability" may be considerably and beneficially reduced for those drugs which undergo hepatic clearance.

Constant Dialysate Concentration

The behavior of the model equations is shown by two types of simulations. In the first simulation, the peritoneal C is held constant, eg, by constant flow dialysis. Then, for constant parameters, equation [1] may be solved to yield:

$$\frac{C_p}{C} = \frac{PA}{R(PA)+k}\left[1 - e^{-\frac{R(PA)+k}{V_D}t}\right]. \quad [3]$$

Equation [3] expresses the relative plasma C (compared with dialysate) as a function of time and two groups of parameters. The first group, $PA[R(PA)+k]^{-1}$, gives the ratio at steady state. For R = 1, it shows that the C in the plasma will reach a value much less than the C in the dialysate if PA is much less than k. The second group, $V_D[R(PA)+k]^{-1}$, provides a measure of the time of the transient required to approach steady state.

The peritoneal P has not been studied thoroughly for anticancer drugs in man. Values may be estimated from transport studies of other molecules. These studies have been conducted almost exclusively to support peritoneal dialysis for uremia or drug intoxication and to elucidate the transport characteristics of the peritoneal membrane compared with available synthetic hemodialysis membranes. The molecular species used are water soluble with a range of molecular weights, including both indicators of azotemia and marker molecules. Figure 2 shows PA as a function of molecular weight. It is a log-log plot of the form used by Babb et al (2) and includes data from Babb and more recent data from Popovich et al (3). Figure 2 shows that the P is roughly inversely proportional to the square root of molecular weight over the range of

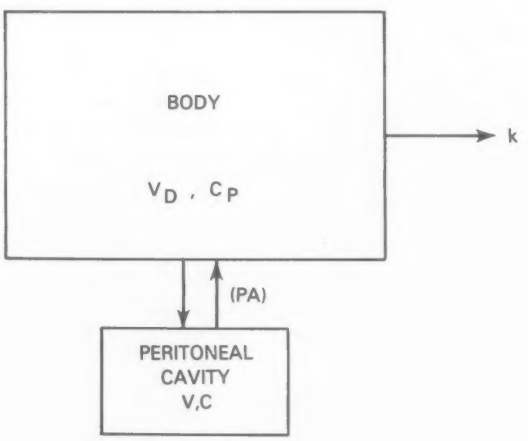

FIGURE 1.—2-compartment open model for peritoneal pharmacokinetics. V_D = volume of distribution; C_p = drug concentration; V = volume; C = concentration; A = area; P = permeability.

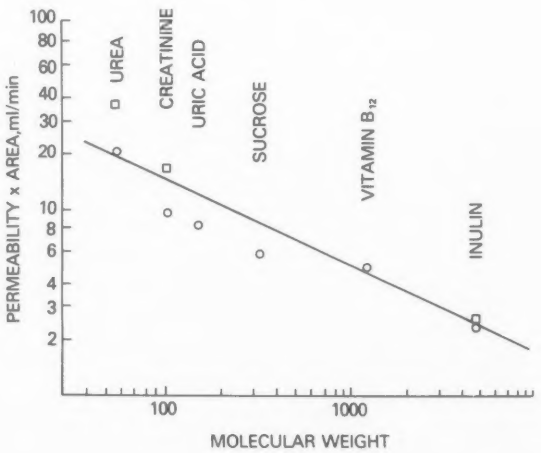

FIGURE 2.—Peritoneal permeability × area vs molecular weight.

interest. Values are considerably less than whole-body clearances of many anticancer drugs.

The credibility of applying figure 2 to estimation of the PA product for water-soluble and lipid-insoluble anticancer drugs is supported by the very limited clinical data that are available. W. A. Bleyer (personal communication) performed peritoneal dialysis on a patient who developed acute kidney failure during high-dose methotrexate (MTX) treatment and obtained a clearance of 6 ml/minute. We have calculated PA products from the data of Clarkson et al (4) and obtained values of 29 ml/minute (7–51 ml/minute) for 5-fluorouracil (5-FU) in two patients and 13 ml/minute (6–25 ml/minute) for 5-FUdR in four patients.

Calculated time courses of plasma C following initiation of constant-C dialysis are shown in figure 3 for a range of parameters. The V_D is taken as total body water (40 liters for a 70-kg adult). For the upper three curves, the elimination constant is taken as inulin clearance (131 ml/minute) (5). The equilibrium distribution ratio is taken to be unity. With these parameters, steady-state plasma Cs are 3%–11% of dialysate C, and the half-life for approach to steady state is about 3–4 hours.

A much smaller ratio of plasma C to dialysate C is calculated if the body clearance is 1 liter/minute (two lower curves). These range from slightly < 1% for PA = 8 ml/minute to 0.2% for PA = 2 ml/minute. The lower value would also represent the behavior of a drug which has PA = 8 ml/minute but is 75% inactivated in the portal circulation by a hepatic first-pass effect. The transient time to reach steady state is significantly reduced compared with the set of simulations at k = 131 ml/minute.

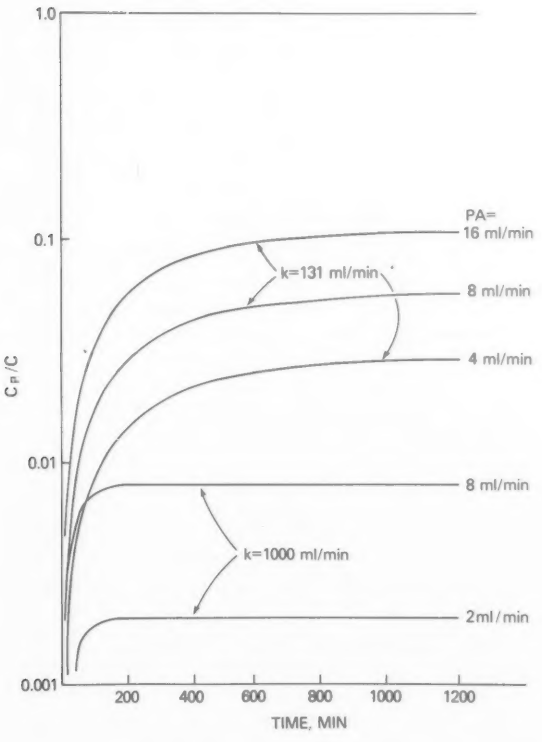

FIGURE 3.—Ratio of plasma C to peritoneal fluid C when the latter is held constant. R = 1, V_D = 40 liters.

Declining Dialysate Concentration

For practical reasons, intermittent peritoneal dialysis may be preferable to continuous dialysis; therefore, we solved equations [1] and [2] simultaneously to yield:

$$\frac{C_P}{C_0} = \frac{PA}{V_D}\,\frac{e^{-\alpha t}-e^{-\beta t}}{\beta-\alpha} \qquad [4]$$

$$\frac{C}{C_0} = \left[\frac{R(PA)+k}{V_D}-\alpha\right]\frac{e^{-\alpha t}}{\beta-\alpha} - \left[\frac{R(PA)+k}{V_D}-\beta\right]\frac{e^{-\beta t}}{\beta-\alpha} \qquad [5]$$

where C_0 is the initial dialysate C and α and β are the two solutions to the following pair of simultaneous equations,

$$\alpha+\beta = \frac{PA}{V}+\frac{R(PA)}{V_D}+\frac{k}{V_D}$$

$$\alpha\beta = \frac{(PA)k}{VV_D}.$$

The solution is a minor variation on the well-known linear, two-compartment open model (6,7). Simulations of plasma and dialysate Cs based on equations [4] and [5] are shown in figure 4 for PA = 8 ml/minute, k = 131 ml/minute, V = 4 liters, V_D = 40 liters, and R = 1. Half of the administered drug is absorbed in 6 hours, and the plasma C, which peaks at about 6½ hours, remains a full log lower than the dialysate C.

Comments

The simulations presented above are encouraging in the order-of-magnitude C ratios. C differences of 1–3 logs could be anticipated, possibly even more for a drug that is almost completely inactivated on a single pass through the liver.

Certain assumptions explicit or implicit in the

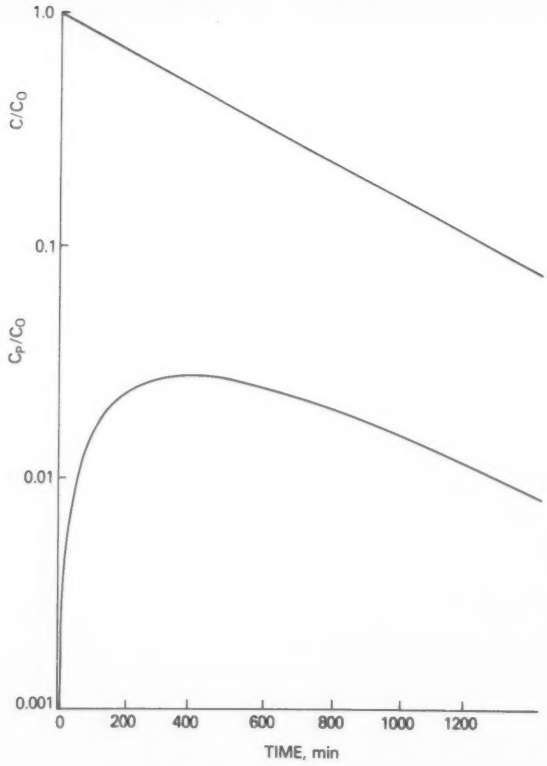

FIGURE 4.—Ratio of plasma C and peritoneal fluid C to initial peritoneal C following single ip dose. R = 1, k = 131 ml/min, PA = 8 ml/min, V = 4 liters, V_p = 40 liters.

prior discussion should be emphasized. First, peritoneal transport parameters have not been published for any anticancer drug. Ps, therefore, must be estimated from what is known about other drugs, endogenous metabolites, or marker molecules. This would seem to be reasonable as long as relevant physical properties of the anticancer drug are similar to those of the other substances. Figure 2 should not be used to estimate PA for a molecule with any significant lipid solubility because transcellular transport mechanisms may greatly increase the rate of diffusion compared with the expected value if P is limited by capillary junctions. Second, the peritoneal P is quite variable. It is known to be affected by drugs (8) and may be altered in disease states. Third, the effective area may be a function of V, but the function is not known. Therefore, values estimated from figure 2, which were obtained at Vs typical of peritoneal dialysis, may differ somewhat from those appropriate for large-volume dialysis.

From the above discussion, those properties which would make a drug appropriate for ip administration become apparent. First, the drug should be capable of killing tumors either directly or by metabolic activation within tumor tissues. A drug such as cyclophosphamide, which requires hepatic activation, would not likely be of unique benefit when administered ip. The drug should possess a low peritoneal PA and should be rapidly cleared from the plasma. Finally, it would be advantageous if antidotal agents were available for iv use which also possessed poor peritoneal P.

PHARMACOKINETIC SIMULATIONS

Based on the encouraging results from the simplified analysis above, we have conducted more detailed pharmacokinetic simulations to serve as a reference for design of clinical protocols and to illustrate a range of expected behavior. These simulations were obtained from modifications of physiologic pharmacokinetic models which we have developed in the course of our investigations of the distribution and disposition of MTX and cytosine arabinoside (ara-C). These two drugs were chosen because they illustrate the behavior which might be expected from a drug cleared predominately via the kidneys (MTX) and one in which both renal clearance and drug metabolism play a role (ara-C).

MTX

Figure 5 is a flow diagram of the model which was used to simulate plasma and peritoneal Cs. The model was originally developed to quantitate species differences in MTX pharmacokinetics with extensive experimentation only in the mouse and the rat (9). The model has been revised on the basis of subsequent transport studies in the rat to incorporate a skin compartment and to permit membrane-limited transport to occur in the bone marrow and gastrointestinal tract (10). Such transport has no significant effect on the systemic distribution of MTX relevant to peritoneal drug administration; however, the details have been retained in the model because the bone marrow and gastrointestinal tract are important sites of toxicity which might be examined in the context of the model as appropriate human parameters become available. In addition to the above revisions, the biliary clearance has been reduced. It appears, but has not been fully documented, that biliary secretion is a much less significant route of elimination in man than in mice and rats. Creaven et al (11) obtained only

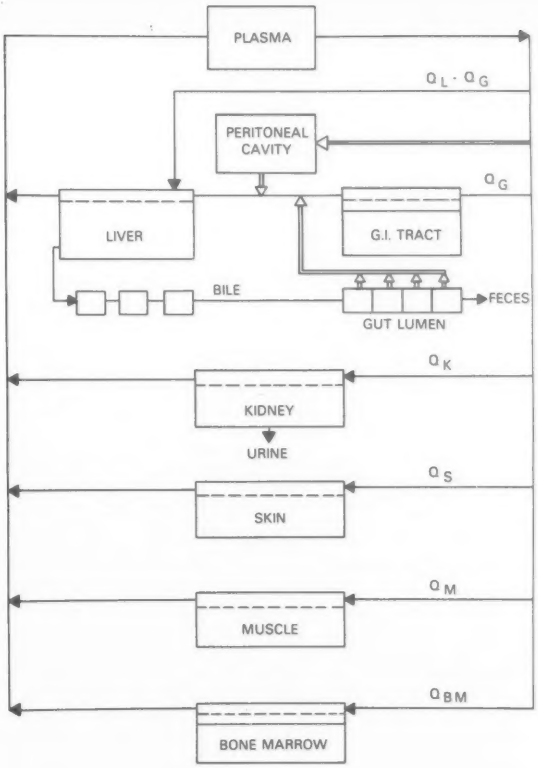

FIGURE 5.—Schematic of pharmacokinetic model for MTX. Light double lines represent mass transfer. Single lines represent flow.

0.41% of administered MTX in 48 hours from one patient with a complete biliary fistula. Studies of fecal elimination have generally shown little radioactivity derived from iv doses. Henderson et al (12) obtained 0–4% (mean, 2%) in 48 hours while Huffman et al (13) obtained a range of 0–9% (mean, 1.3%) in 96 hours. Because biliary clearance has not been studied extensively, its significance cannot be ruled out in all patients. Quantitative aspects of enteric disposition of MTX such as residence time, reabsorption, and bacterial metabolism are not well understood. These may differ considerably from patient to patient, and even relatively small amounts of MTX in the gastrointestinal tract could serve as a depot of clinical significance following high-dose protocols.

The model has been further modified for the present simulation by incorporation of a peritoneal cavity. The PA product is estimated from figure 2 at about 8 ml/minute. Since MTX has been reported to be bound to the extent of about 50% (12), the equilibrium distribution ratio between peritoneal dialysate, which is essentially free of proteins, and plasma should be about 0.5.

The mathematic model based on the flow diagram in figure 5 is a set of differential equations which express mass balances on each compartment similar to equations [1] and [2]. A very large set of equations is required to include all effects such as nonlinear binding to tissues and saturable transport. The set of equations and the parameters used for this simulation are included in the Appendix.

The equations were solved numerically by a digital computer to obtain the time course of MTX C in plasma and the peritoneal cavity for a simulated treatment schedule in which 4 liters of solution containing 10^{-5} M MTX is infused into the peritoneal cavity and allowed to remain in the cavity until the 4-hour point after start of infusion. At 4 hours and at subsequent 4-hour intervals for a total of 32 hours, the peritoneal cavity is drained and 4 liters containing 10^{-5} M MTX is added. The initial V and the residual V after drainage are taken as 500 ml.

Figure 6 shows computer simulations based on the model equations. Peritoneal concentration undergoes the expected saw-tooth pattern with approximately 30% of the infused drug absorbed between changes. The plasma C exhibits a pharmacologic plateau effect which is essentially attained within about three exchanges, and the mean C is 25-fold less than the mean peritoneal C.

For ease of computer programming certain approximating simplifications were introduced into the calculations. The peritoneal fluid V was kept at 4.5 liters throughout the calculations. The drain time was neglected. Infusion of MTX at the beginning of each cycle was effected by slowly increasing the amount of drug in the peritoneal cavity over a 30-minute period, as if by injection of a concentrated solution of negligible volume. During the drain and fill time, much of the patient's peritoneal surface would not be bathed in drug at high C. This effect is not shown on the figure, but the possible effect of intermittent reduction of C to a lower value should be considered.

The expected limited P of the peritoneum to citrovorum factor may confer a therapeutic advantage. If citrovorum factor were infused iv at some time during MTX therapy in order to achieve a systemic level equal to the mean MTX C in the plasma, the level in the peritoneal cavity would be expected to achieve peak levels about 70-fold less than peritoneal MTX. The above calculation is based on an assumed PA of 8 ml/minute for citrovorum factor. The above qualification concerning the drain and fill cycle is pertinent here also.

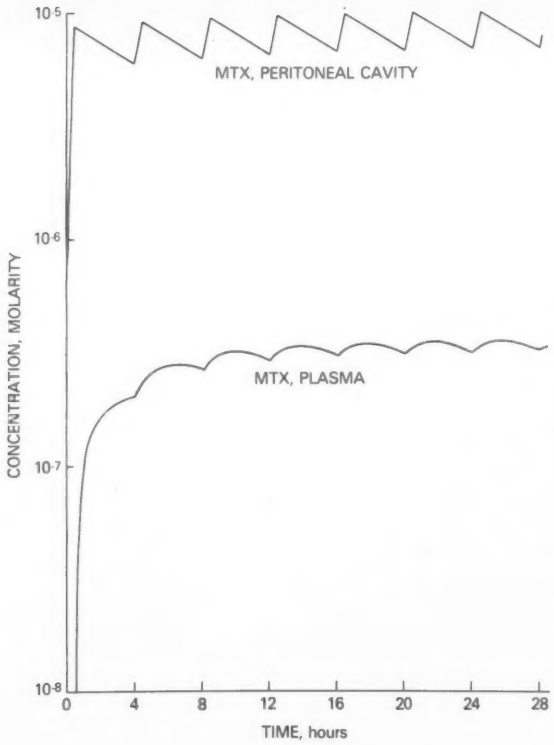

FIGURE 6.—Model simulations of plasma and peritoneal fluid Cs of MTX administered by repeated peritoneal lavage to 70-kg human: V = 4.5 liters (4-liter exchange), PA = 8 ml/min, k = 190 ml/min. Model equations and other parameters are included in the Appendix.

Ara-C

Ara-C is the prototype of a kind of drug which might be usefully employed for intraperitoneal disease. It must be phosphorylated as a requirement for its action (14). It is rapidly inactivated by deamination in man. Wan et al (15) obtained a mean metabolic clearance of 845 ml/minute. In addition, it is cleared by the kidney at a rate approximately equal to creatinine clearance (15). Camiener and Smith (16) showed high levels of pyrimidine nucleoside deaminase activity in human liver with lesser amounts in the kidneys and heart; therefore, one might expect a significant first-pass effect for that part of the absorbed drug which enters the portal circulation. Our calculations (17) suggest that about 80% of the drug that reaches the liver may be metabolized if the C in liver water is significantly less than the Michaelis constant of 1.2–1.6 $\times$ 10^{-4} M (34 μg/ml) (18).

We have modified a pharmacokinetic model developed for ara-C and its deamination product, uracil arabinoside (ara-U) (17), to include a peritoneal cavity as shown in figure 7. The model is physiologic and includes saturable deamination reactions in the liver, heart, and kidneys. Equations and parameters are identical to those described previously except for the inclusion of a balance equation of the form of equation [2] in which R = 1 and the PA product is estimated at 10 ml/minute from figure 2.

The set of model equations was solved numerically for a repetitive peritoneal lavage cycle similar to that described for MTX. As a further computational simplification, the time for infusion of ara-C in each cycle was taken to be negligibly short. The ara-C C in the infusate was taken to be 100 μg/ml. Results for ara-C in the peritoneal fluid and for ara-C and ara-U in the blood are shown in figure 8. It is observed that the ara-C C in the peritoneal fluid undergoes the characteristic saw-tooth pattern, while the ara-C C in the blood attains a pharmacologic plateau within one exchange. Ara-U continues to rise for several exchanges because it is cleared only by the kidneys and, as discussed previously, the transient is related to the ratio of the clearance to the V_D.

The model simulation indicates about 3 logs difference between the C in the peritoneal fluid and the C in the blood. Most of this difference occurs because total clearance is about 2 logs higher than the estimated PA product; however, a significant factor (about 5) is introduced by the calculated first-pass effect. In the model, all drug absorbed from the peritoneal cavity enters the portal system. Anatomically, the parietal peritoneum and lymph drain into the systemic circulation. We have assumed that the fraction of drug absorbed by these routes is small compared with absorption through the visceral peritoneum, mesentery, and omentum. That assumption should be examined closely if first-pass elimination is a critical condition of treatment.

Ara-C exhibits nonlinear kinetics of metabolism. This is pertinent to both the magnitude of the first-pass effect and the metabolic clearance. Since the Michaelis constant determined in vitro is about 34 μg/ml, quite high C_ps would be required before saturation would occur. The consideration of nonlinear elimination kinetics would be extremely important if therapeutic levels are sought in the same range as the Michaelis constant or higher because drug clearance would decrease markedly with increases in C_p.

DISCUSSION

A notable and successful example of local drug therapy occurs in the central nervous system,

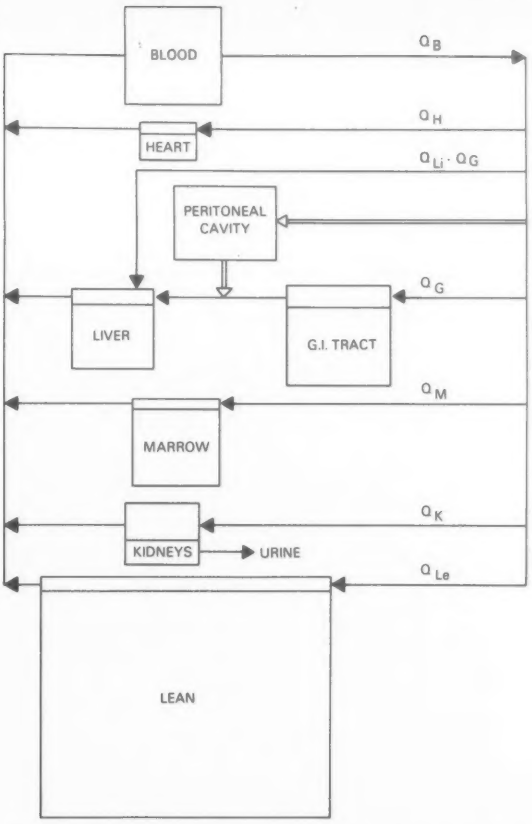

FIGURE 7.—Schematic of pharmacokinetic model for ara-C. Light double lines represent mass transfer. Single lines represent flow.

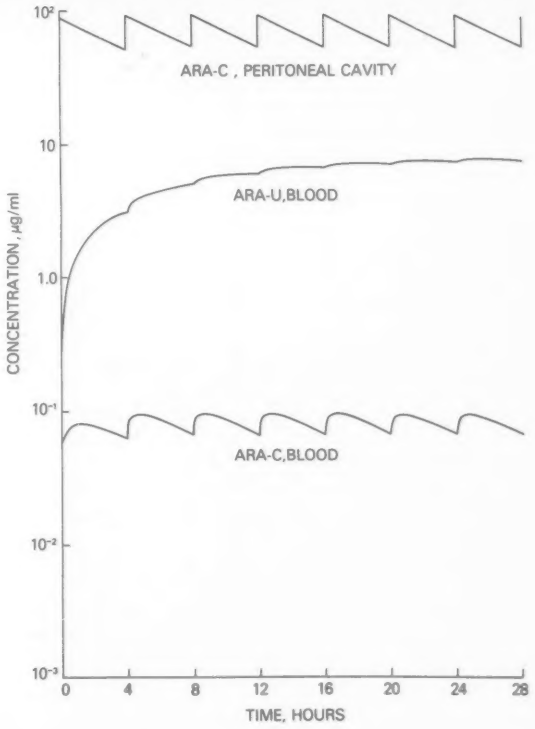

FIGURE 8.—Model simulations of ara-C and ara-U Cs during ara-C administration by repeated peritoneal lavage to a 70-kg human. V = 4.5 liters (4-liter exchange), PA = 10 ml/min. Model equations and other parameters are described by Dedrick et al (17).

where intrathecal drugs are commonly used in the treatment of leukemia. This example is kinetically analogous to ip administration because of restricted transfer of many drugs from the cerebrospinal fluid. It has been shown that intrathecally administered MTX produces a C in the cerebrospinal fluid that is 2 logs higher than the co-existing plasma Cs (19,20). The magnitude of this C difference is fully rationalized by a pharmacokinetic analysis of the transport processes involved (21).

5-FU, an agent known to be moderately effective against ovarian cancer, is rapidly eliminated via renal excretion and metabolic degradation. Data showing very large C differences between ascites and plasma have been reported following intracavity injection of 5-FU or 5-FUdR (4). These were of the order of 3 logs in patients with 1½–3 liters of ascites. They report impressive response to 5-FU and 5-FUdR following intracavity injection when large numbers of free-floating cells were present. Beneficial effect was generally transitory because the patients had disseminated disease. The detailed pharmacokinetics of 5-FU are not as well understood as the pharmacokinetics of ara-C. Elimination and conversion of 5-FU in man are extremely rapid. Finn and Sadée (22) report a half-life of 12 minutes in three patients who received 15 mg/kg iv. Oral administration of the same dose resulted in greatly reduced areas under the plasma C-time curves in two subjects but not in a third. Finn and Sadée suggest a hepatic first-pass effect on systemic bioavailability and a dependence of anabolic metabolites on the rate of absorption. They infer saturation of degradative metabolism diverting a larger fraction of 5-FU into the anabolic pool in rats and feel that this may relate to a disproportionate increase of toxicity observed clinically. Clarkson et al (4) found that 5-FU is degraded more completely after oral

administration or continuous infusion than following a rapid iv dose, possibly indicating saturation of catabolic enzymatic pathways. Continuous infusion into the hepatic artery, however, resulted in similar plasma Cs and amounts excreted in the urine compared with continuous iv injection.

Although the above analysis does suggest that exploitable C differences are attainable, ip administration of cancer drugs has a long and not uniformly successful history (23). In particular, a clinical trial of ip hemisulfur mustard (24) did not reveal any advantage for this form of therapy in the treatment of ovarian cancer. We would, however, suggest that past clinical trials of ip drug therapy may have failed for reasons which are both explainable and avoidable. None of the clinical trials utilizing this approach have adhered to the following guidelines:

a. Tumors should have small thickness.
b. Large Vs of drug-containing solution should be used to ensure exposure of entire peritoneal surface.
c. Care should be exercised to avoid co-administration of drugs which might lessen either hepatic or renal clearance of the drug in question.
d. The ratio of plasma clearance to peritoneal P should be high.

The first point is that penetration of drug from the periphery of a tumor toward its center will almost certainly be limited and dependent on the diffusibility of the drug, the blood perfusion of the tumor, and the distance involved. Thus, the effectiveness of ip drug administration is likely to decrease as the tumor size increases. The ideal group of patients in which to test this route of drug administration would be those patients with presumed microscopic residual ovarian cancer, after successful local or systemic therapy, who have a high risk of relapse, not those with bulky tumors. The second point is that with diffuse intra-abdominal disease such as ovarian cancer, it is imperative that the drug be instilled with a sufficient V to ensure even exposure of the entire peritoneal surface. The third point is that the success of this approach is dependent upon plasma clearance being in considerable excess of the peritoneal clearance. Attention must therefore be given to the preservation of plasma clearance. If renal clearance predominates, patients should have intact renal function. Because tubular secretion may play a role in the renal clearance of drugs such as MTX, co-administration of other drugs capable of competing for this tubular transport should be avoided. In a similar fashion, if hepatic clearance is important, drugs competing for this clearance mechanism should be avoided.

We cannot exclude a priori the possibility that local toxicity, eg, to the gastrointestinal tract, may result from ip administration of some drugs at high C. Because of this, and because so many variables are involved, it would seem advisable to carry out such a treatment protocol only under conditions where toxicity and both peritoneal and plasma drug levels can be monitored continually.

In summary, we have presented the pertinent pharmacokinetic theory underlying ip drug administration and have shown that it should be possible to attain a potentially exploitable C difference between the peritoneal and plasma compartments. We have also reviewed those conditions which are likely to optimize this form of therapy and pointed to that patient population most likely to benefit from this form of therapy. Two of the drugs analyzed, 5-FU and MTX, are known to be effective in this disease, and it would appear that either one would make a reasonable subject for a clinical trial.

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APPENDIX.—Model equations and parameters for MTX pharmacokinetics

BONE MARROW:

Extracellular, $$V_{BE}\frac{dC_{BE}}{dt} = Q_B(C_p - C_{BE}) - \frac{k_B V_B C_{BE}}{K_B + C_{BE}} + \frac{k_B V_B C_{BI}}{K_B + C_{BI}},$$

Intracellular, $$V_{BI}\frac{dC_{BI}}{dt} = \frac{\frac{k_B V_B C_{BE}}{K_B + C_{BE}} - \frac{k_B V_B C_{BI}}{K_B + C_{BI}}}{1 + \frac{a_B \epsilon_B}{(\epsilon_B + C_{BI})^2}}, \quad V_B = V_{BE} + V_{BI},$$

KIDNEY: $$V_K\frac{dC_K}{dt} = Q_K(C_p - C_{VK}) - k_K C_p,$$

$$C_{VK} = \frac{1}{2}\left[\sqrt{\left(\frac{a_K - C_K}{R_K} + \epsilon_K\right)^2 + \frac{4\epsilon_K C_K}{R_K}} - \left(\frac{a_K - C_K}{R_K} + \epsilon_K\right)\right],$$

LIVER: $$V_L\frac{dC_L}{dt} = (Q_L - Q_G)(C_p - C_{VL}) + Q_G(C_{GE} - C_{VL}) + PA(C_{pc} - R_{pc}C_p)$$

$$+ \frac{1}{4}\sum_{i=1}^{4}\frac{k_G V_{GL} C_i}{K_G + C_i} - k_L C_{VL},$$

$$C_{VL} = \frac{1}{2}\left[\sqrt{\left(\frac{a_L - C_L}{R_L} + \epsilon_L\right)^2 + \frac{4\epsilon_L C_L}{R_L}} - \left(\frac{a_L - C_L}{R_L} + \epsilon_L\right)\right]$$

BILE DUCTS: $$\frac{dr_1}{dt} = \frac{k_L C_{VL}}{\tau} - \frac{r_1}{\tau}, \quad \frac{dr_2}{dt} = \frac{r_1 - r_2}{\tau}, \quad \frac{dr_3}{dt} = \frac{r_2 - r_3}{\tau},$$

MUSCLE: $$V_M\frac{dC_M}{dt} = Q_M(C_p - C_{VM}),$$

$$C_{VM} = \frac{1}{2}\left[\sqrt{\left(\frac{a_M - C_M}{R_M} + \epsilon_M\right)^2 + \frac{4\epsilon_M C_M}{R_M}} - \left(\frac{a_M - C_M}{R_M} + \epsilon_M\right)\right]$$

GUT TISSUE:

$$\text{Extracellular,} \quad V_{GE}\frac{dC_{GE}}{dt} = Q_G(C_p - C_{GE}) - \frac{k_{GT}V_{GT}C_{GE}}{K_{GT}+C_{GE}} + \frac{k_{GT}V_{GT}C_{GI}}{K_{GT}+C_{GI}} ,$$

$$\text{Intracellular,} \quad V_{GI}\frac{dC_{GI}}{dt} = \frac{\frac{k_{GT}V_{GT}C_{GE}}{K_{GT}+C_{GE}} - \frac{k_{GT}V_{GT}C_{GI}}{K_{GT}+C_{GI}}}{1 + \left(\frac{a_G\epsilon_G}{\epsilon_G+C_{GI}}\right)^2} ,$$

$$V_{GT} = V_{GE} + V_{GI} ,$$

GUT LUMEN:

$$\frac{1}{4}V_{GL}\frac{dC_1}{dt} = r_3 - k_F V_{GL}C_1 - \frac{1}{4}\left(\frac{k_G V_{GL}C_1}{K_G+C_1}\right) ,$$

$$\frac{1}{4}V_{GL}\frac{dC_i}{dt} = k_F V_{GL}(C_{i-1} - C_i) - \frac{1}{4}\left(\frac{k_G V_{GL}C_i}{K_G+C_i}\right), \; i = 2,3,4 ,$$

$$\frac{dC_{GL}}{dt} = \frac{1}{4}\sum_{i=1}^{4}\frac{dC_i}{dt} ,$$

PERITONEAL CAVITY:

$$V_{pc}\frac{dC_{pc}}{dt} = PA(R_{pc}C_p - C_{pc}) ,$$

PLASMA:

$$V_p\frac{dC_p}{dt} = Q_B C_{BE} + Q_K C_{VK} + Q_L C_{VL} + Q_M C_{VM} + Q_S C_S - (Q_B + Q_K + Q_L + Q_M + Q_S)C_p ,$$

SKIN:

$$V_S\frac{dC_S}{dt} = Q_S(C_p - C_S) ,$$

MODEL PARAMETERS FOR A 70-KG HUMAN

	Plasma	Muscle	Kidneys	Liver	Skin	Gut tissue ECF	Gut tissue ICF	Gut lumen	Bone marrow ECF	Bone marrow ICF
Volume, V (ml)	3000	30,000	280	1350	7800	700	1400	2100	280	1120
Flow rate, Q (ml/min)	—	420	700	800	78	700	—	—	72	—
Linear distribution ratio, R	1	0.15	3.0	3.0	1.0	1.0	—	—	1.0	—
Strong binding constant, a (μg/ml)	0	0	0.3	0.4	0	0	0.2	0	0	0.2
Dissociation constant, ϵ (μg/ml)	—	—	0.00001	0.00001	—	—	0.00001	—	—	0.00001
Clearance (ml/min)	—	—	190	10	—	—	—	—	—	—
Transient time for bile or gut lumen (mins)	—	—	—	30	—	—	—	1000	—	—
Maximum facilitated transport, k [μg/(min)(ml)]	—	—	—	—	—		0.014	0.9		0.012
Michaelis constant, K (μg/ml)	—	—	—	—	—		0.45	200		0.9

Separation and Identification of Impurities in Parenteral Methotrexate Dosage Forms [1,2]

Charles E. Hignite, Danny D. Shen, and Daniel L. Azarnoff [3,4,*]

SUMMARY

Eleven lots of parenteral methotrexate (MTX) dosage forms from two manufacturers have been analyzed and found to be 93.4% ± 2.1% (SD) pure by a new procedure employing a DEAE-cellulose column adapted for high performance liquid chromatography instrumentation. In addition to 4-amino-10-methylpteroic acid and N^{10}-methylpteroylglutamic acid, which were previously identified contaminants, and benzyl alcohol, which was added as a preservative, we have also isolated and identified 2,4-diamino-6-methylpteridine, 2,4-diamino-6-bromomethylpteridine, and *p*-methylaminobenzoic acid. Another impurity has tentatively been identified as 4-amino-10-methylpteroic acid amide. The individual impurities vary from 0.1% to 4.8% of the ultraviolet absorbing material which elutes from the column. Additional impurities were detected by gas chromatography-mass spectrometry. Since "high-dose" therapy may involve the administration of up to 20 g of MTX/day, the effect of the impurities present in these dosage forms on the course of the patient's therapy should be determined.

[Cancer Treat Rep 62:13–18, 1978]

Methotrexate (MTX) is an effective antineoplastic agent which has been widely used both alone and in combination with other agents (1,2). MTX competitively inhibits the conversion of folic acid to folinic acid (N^5-formyl tetrahydrofolate, citrovorum factor) but its affinity for the enzyme dihydrofolate reductase is so much greater than folic acid that large doses of the latter do not reverse the action of MTX. The mechanism of action (3) and pharmacokinetics (4) of MTX have been reviewed. MTX has until recently been used in small doses (30 mg/m^2). Since the action of MTX can be acutely circumvented by administration of folinic acid, Djerassi et al administered massive doses (up to 20 g of MTX/day) and observed that previously resistant tumors responded to treatment (5).

The use of large doses of MTX necessitates a closer examination of the purity of the commercial preparations. Indeed, USP standards (6) only require that the material contain not < 85% of $C_{20}H_{22}N_8O_5$ calculated on the anhydrous basis. Thus, it is possible that as much as 1.5 g of impurities might be given with a 10-g dose. The role that these impurities play in the undesirable effects of MTX (oral and gastrointestinal tract ulceration, bone marrow suppression, renal insufficiency, and immunosuppression) is unknown, but may be important. In individuals receiving high doses of MTX, renal insufficiency has been noted with increasing frequency (7).

Tong et al (8) have previously separated a few impurities from MTX by high performance liquid chromatography (HPLC) using a reverse phase column, but only two were identified and none were quantitated. We have adapted the commonly used method of ion-exchange chromatography on DEAE cellulose (9–11) to HPLC and, by utilizing gas liquid chromatography (GC) and mass spectrometry (MS), we have separated and identified most of the impu-

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[4]We are indebted to Kenneth Shearer for his expert technical assistance.

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rities which are present at levels > 0.1% in the parenteral dosage form of MTX that are detected by ultraviolet (UV) absorbance. Identifications were made by comparison of the properties of the unknown compounds with authentic standards.

METHODS

Six different lots (484-356, 481-490, 467-160, 484-362, 425-205, and 449-241) of a parenteral dosage form of MTX (50 mg/2 ml vials, Lederle Laboratories, Pearl River, NY) were obtained over a 2-year period from the University of Kansas Medical Center and Kansas City Veterans' Administration Hospital pharmacies. We also obtained five additional lots (76-220, 76-227, 77-211, 77-220, and 77-223) prepared by Ben Venue Laboratories (Bedford, Ohio). Samples of folic acid (Schwarz/Mann, Orangeburg, NY), *p*-methylaminobenzoic acid, *p*-aminobenzoic acid, and benzyl alcohol (Aldrich Chemical Co, Milwaukee, Wis) were used as obtained from the manufacturers. The agents used for derivatization, which included trimethylchlorosilane (PCR Inc, Gainesville, Fla), *N,O*-bis-(trimethylsilyl)trifluoroacetamide (Regis Chemical Co, Morton Grove, Ill), and *N*-methyl-*N*-nitrosourea (K & K, Cleveland, Ohio) were also used without purification. Samples of pure N^{10}-methylpteroylglutamic acid and 4-amino-10-methylpteroic acid were generous gifts of Lederle Laboratories.

Separations were achieved using a Varian high pressure liquid chromatograph, Model 8500 (Palo Alto, Calif), equipped with a 0.9-cm internal diameter × 25-cm DE52 DEAE-cellulose column (Whatman Ltd, Clifton, NJ) fitted with 5μ inlet and outlet filters. Samples corresponding to 1–5 mg of commercially available MTX were injected via a 100-μl loop injector (Rheodyne, Berkeley, Calif). Two ammonium bicarbonate buffers at pH 8.3 were used for the mobile phase. A 0.1 M solution was used initially and this was changed linearly at a rate of 0.5%/minute to a 0.4 M solution. The volatile ammonium bicarbonate buffer was used to facilitate removal from the eluted fractions which were subsequently further analyzed by thin-layer chromatography (TLC) and MS. A pressure of < 100 atmospheres was required to maintain a flow rate of 100 ml/hour through the column. Flow rates > 100 ml/hour should not be attempted or cellulose particles will be forced from the column into the flow cell. The effluent was monitored at either 254 or 280 nm with a Varian 635 variable wavelength UV detector.

All mass spectra were obtained by electron impact (70 eV) and were recorded using a Finnigan 3300 mass spectrometer and a 6000 data system (Sunnyvale, Calif). Compounds were introduced into the ion source either by direct probe or GC inlet. The GC column used was 6 feet × 1/8 inch 3% OV-17 on 100–120 mesh Gas Chrom Q and was programmed from 80°C to 320°C at 12°C/minute using approximately 30 ml/minute of helium as the carrier gas. Samples were either methylated with diazomethane in methanol at room temperature or trimethylsilylated (TMS) with a 1:1:1 mixture of trimethylchlorosilane, *N,O*-bis-(trimethylsilyl)trifluoroacetamide, and pyridine at 60°C for 10 minutes. Details concerning the mass spectral fragmentation of these compounds have been published elsewhere (12).

Preparative TLC was carried out using 20 × 20 cm × 2 mm silica gel G plates with fluorescent indicator ("Uniplates," Analteck, Newark, Del). Water or methanol was used as the developing solvent. Bands were eluted with ethanol:water (1:1) and then analyzed by the DEAE-cellulose column to correlate the TLC band with HPLC peak. TLC of the dosage form was used primarily to obtain sufficient amounts of some of the impurities for identification.

The amount of material in each peak was quantitated by measurement of the absorbance of the collected HPLC peaks and application of Beer's law using molar extinction coefficients (ϵ) at 254 nm determined with authentic compounds. All measurements were made in ammonium bicarbonate buffer at pH of 8.3 at concentrations between 0.1 and 0.4 M. For purposes of quantitation it was assumed from preliminary data[5] that unknown peak 4 was 4-amino-10-methylpteroic acid amide with ϵ = 20,000 M^{-1} cm^{-1} and that peak 8 had the same ϵ and molecular weight as MTX. The two compounds in peak 2 had the same ϵ and were quantitated without separation.

RESULTS

A typical HPLC analysis using absorbance at 254 nm for detection is shown in figure 1. Eight peaks are easily discernible and were detected in each lot of MTX analyzed. An additional peak appeared between peaks 2 and 3 in one analysis but this was found to be due to contamination of the sample with phthalate plasticizers. In chromatograms obtained measuring absorbance at 280 nm, peak 4 contained one and sometimes two shoulders, indicating the presence of at least two components, and sometimes three. The identity of each of the eight peaks is indicated in table 1 along with the analytic methods used to establish their structures. The percentage

[5]A sample of peak 4 was analyzed by field desorption MS on a MAT-731 MS. We are indebted to Dr. Klaus Biemann of the Massachusetts Institute of Technology for these analyses.

TABLE 1.—Characteristics used to identify impurities*

Analytic method	HPLC peak 1	2	2	3	4	5	6	7	8
	Benzyl alcohol	2,4-Diamino-6-methylpteridine	2,4-Diamino-6-bromomethylpteridine	*p*-Methylaminobenzoic acid	Unknown†	4-Amino-10-methylpteroic acid	MTX	N^{10}-methylpteroylglutamic acid	Unknown
HPLC retention time	+			+		+	+	+	
TLC R_f methanol				+		+	+	+	
TLC R_f water				+		+	+	+	
Probe-MS CH_2N_2		+	+			+	+	+	
GC-MS TMS	+	+	+	+					
GC-MS	+								
Probe-MS		+	+						

* + = impurity has identical characteristics to either authentic sample and/or published spectra (*see* footnote 6).
†Examination by field desorption MS suggests that peak 4 contains 4-amino-10-methylpteroic acid amide.

TABLE 2.—% of total UV absorbance eluted from column*

Lot†	HPLC peak 1	2	3	4	5	6	7	8
L 425-205	—	0.1	0.3	0.5	1.0	93.8	3.9	0.5
L 467-160	—	0.1	0.3	0.3	1.8	92.9	4.3	0.3
L 481-490	—	0.1	0.2	0.6	1.3	92.5	4.8	0.5
L 484-356	—	0.1	0.2	0.5	1.9	93.4	3.6	0.4
L 484-360	—	0.1	0.5	0.1	6.7	89.0	3.2	0.4
L 484-362	—	0.1	0.5	0.4	3.1	91.8	4.1	0.4
BV 76-220	—	0.1	0.4	0.5	0.8	95.6	1.4	1.3
BV 76-227	—	0.4	0.4	1.2	2.2	92.7	1.5	1.6
BV 77-211	—	0.7	0.0	0.5	0.3	95.7	1.0	1.3
BV 77-220	—	0.3	0.8	1.4	2.3	93.2	1.2	0.3
BV 77-223	—	0.2	0.1	0.2	0.3	96.6	0.1	1.1
L, average		0.11	0.35	0.39	2.64	92.22	3.98	0.39
L, SD		0.02	0.13	0.16	2.14	1.75	0.56	0.06
BV, average		0.34	0.33	0.76	1.18	94.74	1.04	1.12
BV, SD		0.21	0.32	0.51	1.00	1.70	0.56	0.49

*Does not include benzyl alcohol.
†L = Lederle; BV = Ben Venue.

each impurity contributed to the total amount of UV absorbing compounds which eluted from the column for each lot of MTX is shown in table 2. MTX from both manufacturers was > 92% pure by this assay. Peak 1, benzyl alcohol, was not included in the calculations since it is an added preservative. We consistently found benzyl alcohol to be nearer 0.5% than the 0.9% wt/vol reported for the Lederle preparation. Losses due to the volatility of this compound may explain the discrepancy.

In addition to analyzing the MTX lots by HPLC, analyses were also carried out by GC-MS on samples of lyophilized lots of MTX which were converted into their TMS derivatives. These analyses (not shown) indicated by comparison with reference mass spectra[6] the presence of small amounts of a plasticizer, tri-(2-butoxyethyl)-phosphate (< 0.05%), and phosphoric acid (< 0.05%) in addition to benzyl alcohol and *p*-methylaminobenzoic acid. Also present were very small amounts (< 0.01%) of three compounds having general structure I and three other compounds having general structure II (fig 2). The first three compounds were assigned partial structure I since their mass spectra all exhibited abundant ions at m/e = 134 and an associated ion at m/e = 106 (loss of CO from m/e = 134). The three

[6]Markey SP, Urban WG, and Levine SP. Mass spectra of compounds of biological interest. Oak Ridge, Tenn, US Atomic Energy Commission. National Technical Information Service.

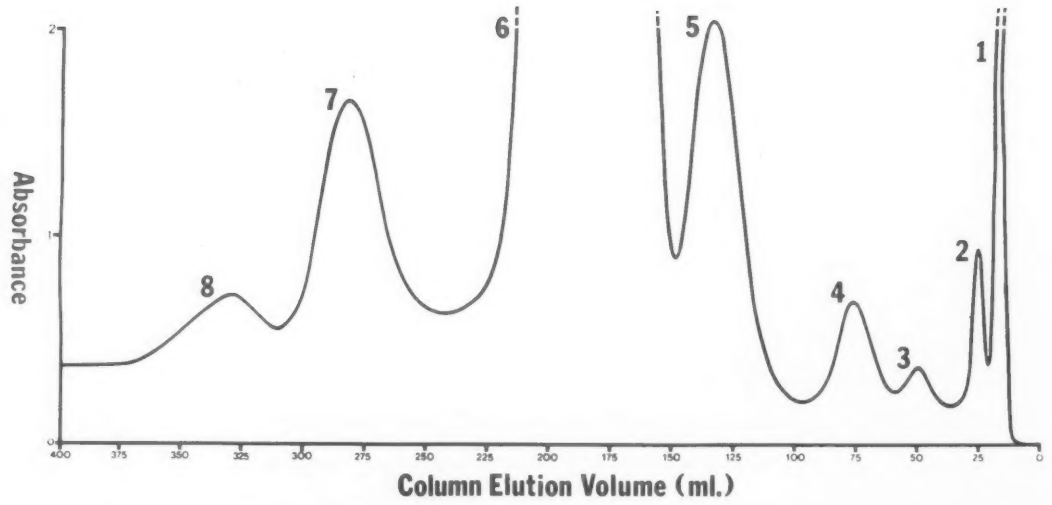

FIGURE 1.—Representative separation of MTX and impurities from a parenteral dosage form. The eluate was monitored for UV absorbing compounds at 254 nm.

latter compounds were assigned partial structure II since their mass spectra exhibited abundant ions at m/e = 176, m/e = 148 (loss of CO from m/e = 176), m/e = 134 (loss of ketene from m/e = 176), and m/e = 106 (loss of CO from m/e = 134).

DISCUSSION

Most of the major contaminants of the commercially available dosage form of MTX have been identified and, in addition, partial structures have been assigned to many of the minor components. Most of the contaminants appear to be byproducts of the synthetic procedure used to prepare MTX. Although we are not aware of the exact procedure(s) used by the manufacturers, the synthesis may well be similar to that used by American Cyanamid for folic acid.[7] There have been several reports (13–16) of additional ways of preparing MTX and its precursors. Substitution of the necessary starting materials such that MTX would be generated instead of folic acid would result in the synthesis outlined in figure 3. The presence of the substituted benzoic acids which include *p*-methylaminobenzoic acid, the three compounds having partial structure I, and the other three compounds having partial structure II are all rationally explained by *p*-methylaminobenzoic acid being a synthetic precursor. A byproduct of the synthesis of 2,4-diamino-6-pteridine carboxaldehyde would be 2,4-diamino-6-bromomethylpteridine which in turn could form 2,4-diamino-6-methylpteridine and could account for the presence of these two compounds. The presence of 4-amino-10-methylpteroic acid may have arisen by either condensation of 2,4-diamino-6-pteridine carboxaldehyde or 2,4-diamino-6-bromomethylpteridine with *p*-methylaminobenzoic acid or hydrolysis of the amide linkage of MTX. N^{10}-methylpteroylglutamic acid is known to be a byproduct of the synthetic procedure (9).

General Structures
(X=unknown residues)

FIGURE 2.—General structures of a series of impurities in MTX partially characterized by GC-MS. *See* Results section for further details.

The procedure we have described using HPLC instrumentation should facilitate both confirmation of the purity of the MTX to be used for chemotherapy or in metabolism studies as well as the isolation of metabolites. Confirmation of the purity of MTX

[7]US patent 2,563,707. Process for preparing pteridines, 1951.

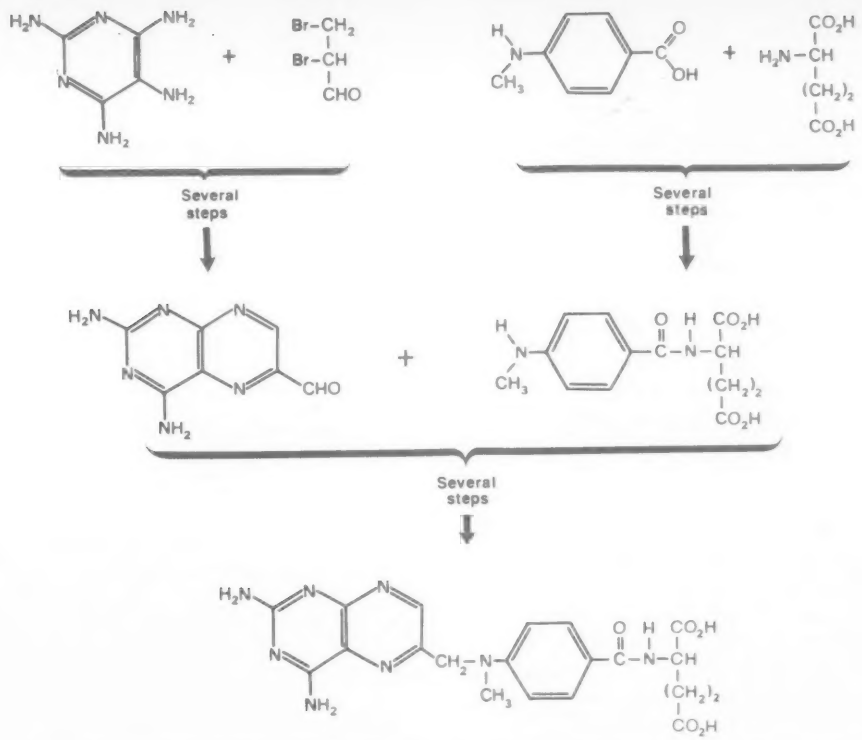

FIGURE 3.—Synthetic pathway which could account for the impurities found in the MTX dosage form.

used for metabolism studies is especially important since some compounds, such as 4-amino-10-methylpteroic acid, are both metabolites of MTX and impurities in the commercially available dosage forms. Most studies concerning the metabolism of MTX (9,10,17–23) have been undertaken with MTX purified by column chromatography on DEAE cellulose and are probably satisfactory.

While we have reasonably well characterized the impurities in the parenteral dosage forms of MTX, the role these impurities play in the therapeutic and/or toxic effects of MTX, especially in high doses, remains to be examined. The recent observation that several of the impurities inhibit dihydrofolate reductase (24) establishes that they are biologically active, but does not necessarily link them with the adverse effects associated with MTX therapy. The clinical role of these compounds should be investigated or they should be removed from the dosage form.

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Chromomycin A_3 for Advanced Breast Cancer: A Southwest Oncology Group Study [1,2]

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SUMMARY

Chromomycin A_3 was administered iv to 26 patients in a phase I trial. The maximum tolerated dose established in this study was 0.75 mg/m²/day iv × 5 days. The drug was then given to 48 evaluable patients with far-advanced disseminated breast cancer. Two short partial remissions and one clinical improvement were seen. Toxic manifestations consisted of frequent and usually reversible renal toxicity, nausea and vomiting, occasional thrombocytopenia, hypocalcemia, and two instances of semicoma. Drug toxicity may have contributed to the death of two patients.

[Cancer Treat Rep 62:19–22, 1978]

Chromomycin A_3 is the most active of all the antitumor antibiotics of the chromomycin group isolated from a culture of *Streptomyces griseus*. Chemically, it is closely related to mithramycin and olivomycin (1). The principal mechanism of action is believed to be inhibition of DNA-dependent RNA polymerase (2–4). In vitro, the antibiotic will only complex with DNA in the presence of bivalent cations, especially Mg^{++}. It is the antibiotic-Mg^{++} complex which appears to attach to DNA guanine (5). The drug is excreted in the bile and slowly in the urine. In mice, the highest concentration is found in the liver, kidneys, and intestine (6). Antitumor activity was demonstrated in Yoshida sarcoma, mouse ascites Sarcoma 180, rat ascites Hepatoma 130, SN36 lymphatic leukemia, and Ehrlich ascites tumor (7). Older studies conducted in Japan and South Africa suggest that chromomycin A_3 is active against a variety of human tumors including breast cancer (8) and colon cancer (9,10). More recently, Moertel et al (11) were not able to confirm the efficacy of chromomycin A_3 in colorectal cancer.

A phase I evaluation of chromomycin A_3 was conducted at Wayne State University. After the maximum tolerated dose was established, the SWOG initiated a phase II study of this drug in advanced metastatic breast cancer. Both phases of the study are reported here.

MATERIALS AND METHODS

From August 1972 until February 1973, 26 evaluable patients received 43 evaluable courses of chromomycin A_3 as a part of the phase I study. All patients had biopsy-proven far-advanced disseminated cancers no longer amenable to standard therapy. All eligible patients had to have wbc counts $> 5000/mm^3$, platelet counts $> 100{,}000/mm^3$, normal BUN and serum creatinine levels, and expected survival of at least 6 weeks. No previous exposure to mithramycin was allowed. The patients represented in the phase I study had the following tumors: lung, 12; gastrointestinal, five; ear, nose, and throat, four; breast, two; melanoma, one; ovary, one; and lymphoma, one.

Evaluation during therapy included monitoring of hematologic values, hepatic and renal function tests, and electrolyte determinations including serum calcium. Chromomycin A_3 doses ranged from

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[3]This study was conducted by the Southwest Oncology Group (SWOG). The following institutions participated in the study: Wayne State University School of Medicine, Detroit, Mich (*B. Samal, E. Caoili, and L. Baker*); University of Arizona Medical School, Tucson (*S. Jones*); Division of Oncology, Henry Ford Hospital, Detroit, Mich (*R. W. Brownlee*); University of Mississippi Medical Center, Jackson (*F. Morrison*); and University of Kansas Medical Center, Kansas City (*B. Hoogstraten*).

**Reprint requests to:* SWOG Operations Office, 3500 Rainbow Blvd, Suite 201, Kansas City, Kan 66103.

TABLE 1.—Chromomycin A_3: phase I evaluation of toxicity

Schedule (mg/m²/day × 5 days)	No. of courses	Toxic effects (No. of courses)*			
		Renal†	Nausea and/or vomiting	Decreased calcium	Decreased magnesium
0.25	5	3	0	—	—
0.50	4	2	0	—	—
0.75	11	7	1 mild 6 moderate	1	1
1.0	8	3	1 mild 2 moderate	—	—
1.25	4	2	1 mild 2 moderate	1	1
0.75‡	9	5	4 moderate	—	—
1.0‡	2	1	—	—	—

*Other toxic effects consisted of enterocolitis in 1 patient at 0.5 mg/m² (multiple superficial ulcerations at autopsy) and local burning and ulcerations from paravenous infiltration.
†Based on alteration of serum creatinine and/or creatinine clearance.
‡Every-other-day schedule.

TABLE 2.—Effect of chromomycin A_3 on 24-hr creatinine clearance

Patient No.	Dose (mg/m²/day × 5 days)	Creatinine clearance (liters/day)	
		Pretreatment	During treatment
1	0.75	90	80.5
2	0.75	164	67.4
3	0.75	91	74.0
4	1.0	68	40.0
5	1.25	120	48.6
6	1.5	69	18.0
7	1.5	191	85.0
8	0.75*	99	105
9	0.75*	99	132

*Every other day × 5 doses.

0.25 to 1.5 mg/m²/day iv × 5 days. Eight patients were treated with an every-other-day schedule in an attempt to minimize renal toxicity.

From May 1974 until February 1975, 52 women with advanced breast cancer were treated with chromomycin A_3 at a starting dose of 0.75 mg/m²/day iv × 5 days repeated at 4-week intervals. In subsequent courses, dose adjustments were based on prior toxicity. Eligibility requirements were similar to those of the phase I study with the additional provision that patients were to have clinically measurable disease.

Response was defined as a ≥ 50% decrease in the sum of the products of the longest perpendicular diameters of measured lesions. Patients with bone involvement as the only metastatic site were classified as having response when lytic lesions showed re-ossification. Stable disease was defined as a steady state or improvement that did not meet the above criteria of response. Increasing disease was defined as at least a 25% increase in measurable lesions or appearance of new lesions.

RESULTS

The phase I study revealed the major toxic effects to be renal and gastrointestinal. Both types of toxicity appeared to be dose-related (table 1). Because of renal abnormalities, 24-hour creatinine clearance was performed prior to and immediately after drug therapy of nine patients. Decreases in clearance were demonstrated in seven of nine patients studied (table 2). Renal toxicity appeared to be reversible in all cases. Gastrointestinal toxic effects were predominantly nausea and vomiting. One patient experienced enterocolitis at Day 10, and at autopsy multiple superficial ulcerations of the colon were demonstrated. One patient experienced

local burning and ulceration as a result of paravenous infiltration.

The only positive response was a 20% reduction in measurable tumor in a woman with breast cancer. The maximum tolerated dose was 0.75 mg/m²/day iv × 5 days, and thus a phase II study was launched.

PHASE II STUDY

Patients.—Fifty-two women with far-advanced disseminated breast cancer were entered into the study. Forty-eight patient records were available for evaluation and four were inevaluable or incomplete. All patients had had at least one previous chemotherapeutic trial. Thirty-seven patients had visceral disease and 30 had clinically demonstrable skeletal metastases, usually quite extensive.

Results.—Only two women experienced a partial remission which lasted 1½ and 3 months respectively. The first patient had a regression of pulmonary metastases and received three courses of chromomycin A_3. The second patient had a response of soft tissue masses and received a total of five courses of chromomycin A_3 before further progression of the tumor was noted. One patient with skeletal metastases had a significant improvement of performance status, decrease in pain, and a reversal of a leukoerythroblastic reaction. She received a total of five courses of chromomycin A_3 before clear-cut progression of her disease became apparent. In three instances the disease stabilized for about 3 months while all the remaining patients had demonstrable progression of their metastases without any intervening subjective or objective improvement. Nine deaths occurring within 4 weeks of the beginning of therapy were classified as treatment failures.

Toxicity.—The toxic effects are listed in table 3. Sixteen patients received one course, 19 received two courses, and 13 received three or more courses of chromomycin A_3. Of the 32 patients who received two or more courses, the dose was escalated in 17 from 0.75 to 0.9 mg/m²/day × 5 days. This was usually done because of lack of either toxicity or antitumor effect during the first course of treatment. In all but three cases the escalation of the dose was associated with some toxicity at the higher dose level. In two instances, chromomycin A_3 may have contributed to the patients' death. One of these patients had pre-existing renal impairment and died of renal failure 14 days after completion of the first course of therapy. The second patient had increasing creatinine prior to death but only pneumonia and liver metastases were found during postmortem examination. Tissue necrosis due to extravasation of the drug was severe in three instances, requiring a skin graft in one patient. Some renal toxicity was seen in 19 patients (table 3). In most patients this toxicity proved to be reversible. Hypocalcemia was seen in five patients. It occurred usually during the 5 days of treatment or within a few days thereafter. In most patients, calcium determinations were not performed frequently enough to estimate accurately the frequency of this phenomenon. Stupor and semicoma were observed in four patients and undoubtedly were due to extensive visceral metastases. In two instances, however, there was a direct temporal relation to the administration of chromomycin A_3. One of these patients lapsed into semicoma on two occasions when exposed to the drug even though the dose was reduced by 50% for the second course of therapy. The second patient did not have any major liver or other visceral involvement to account for this phenomenon.

TABLE 3.—Toxicity of chromomycin A_3 in 48 patients with breast cancer

Toxic effect	No. of patients
Renal*	
Mild	6
Moderate	7
Severe	4
Possibly fatal	2
Total	19
Nausea and/or vomiting	23
Hypocalcemia	5
Skin necrosis	4(3 severe)
Thrombocytopenia (< 100,000 cells/mm³)	5
Leukopenia (3000–4000 cells/mm³)	2
Confusion	3
Semicoma	2
Thrombophlebitis	1

* Mild = < 30% reduction of creatinine clearance, or creatinine of 1.6–2.2 mg/100 ml, or BUN of 20–35 mg/100 ml; moderate = 30%–60% reduction of creatinine clearance, or creatinine of 2.2–3.0 mg/100 ml, or BUN of 35–50 mg/100 ml; severe = worse than moderate, with recovery; possibly fatal = no recovery of renal function before death.

DISCUSSION

The few responses seen in this study were unimpressive. Similarly, a recent report from the Mayo Clinic (11) indicates no significant activity of chromomycin A_3 in 27 patients with colorectal cancer when given in a dose of 0.9 mg/m²/day × 5 days. The more favorable Japanese experience with this

drug (8,12,13) was achieved with substantially lower daily doses given for extended periods of time.

The toxic manifestations of chromomycin A_3 seen in this study confirm previous reports (11,14,15) that renal damage appears to be dose-limiting. Nausea, vomiting, and, rarely, stupor and semicoma may also force discontinuation of chromomycin A_3 therapy. In view of the structural similarity between mithramycin and chromomycin A_3, the hypocalcemia seen in this and other studies (14,15) is of interest and is potentially exploitable in the management of hypercalcemia.

As used in this study, chromomycin A_3 appears to have very limited activity in far-advanced disseminated breast cancer. Exploration of different treatment schedules may still be appropriate.

APPENDIX

No. of evaluable patients	Contributed by—
28	Wayne State University (Detroit, Mich)
8	University of Arizona College of Medicine (Tucson)
6	Henry Ford Hospital (Detroit, Mich)
5	University of Mississippi Medical Center (Jackson)
4	Oklahoma Medical Research Foundation (Oklahoma City)
4	Scott and White Clinic (Temple, Tex)
3	University of Arkansas Medical Center (Little Rock)
3	Wilford Hall USAF Medical Center (Lackland AFB, Tex)
3	University of Kansas Medical Center (Kansas City)
2	Providence Hospital (Southfield, Mich)
2	Keesler USAF Medical Center (Keesler AFB, Miss)
2	Baylor College of Medicine (Houston, Tex)
1	Borgess Medical Center (Kalamazoo, Mich)
1	Brooke General Hospital (Fort Sam Houston, Tex)
1	University of Texas (Galveston)
1	Utah Veterans' Administration Hospital (Salt Lake City)

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Cross-Linking of DNA in L1210 Cells and Nuclei Treated With Cyclophosphamide and Phosphoramide Mustard [1,2]

Y. A. Surya, J. M. Rosenfeld, and B. L. Hillcoat [3,*]

SUMMARY

Phosphoramide mustard, formed from cyclophosphamide in vivo and in vitro, may be the active metabolite of this drug. We have found phosphoramide mustard to be at least 100 times more potent than cyclophosphamide in inhibiting growth of two strains of the L1210 lymphoma in culture. Phosphoramide mustard also produced enlargement of cells, an effect not seen with cyclophosphamide. Phosphoramide mustard significantly increased the amount of cross-linked DNA after incubation with intact LM4 cells or nuclei isolated from these cells. Cyclophosphamide had a similar effect only in the isolated nuclei. These findings strengthen the proposed role of phosphoramide mustard as the active metabolite of cyclophosphamide. The effect of cyclophosphamide on nuclei is unexplained.

[Cancer Treat Rep 62:23–29, 1978]

Cyclophosphamide is a widely used alkylating agent in the chemotherapy of cancer. The drug is nontoxic to cancer cells in vitro and requires activation by the liver in vivo. The nature of the activation process is now reasonably well understood. Recent evidence indicates that cytotoxicity of cyclophosphamide is due to its metabolite, phosphoramide mustard. Both cyclophosphamide and phosphoramide mustard have a β-chloroethyl side chain available for alkylation. Consequently, the difference in their cytotoxicity requires an explanation. Friedman (1) and later Colvin et al (2) suggested that the unesterified hydroxyl group of phosphoramide mustard plays a role in alkylation. At physiologic pH the OH group is ionized, resulting in a negative charge on the oxygen which could be distributed over the molecule, enhancing the basicity of the phosphamide linkage. The tertiary nitrogen would be nucleophilic enough to allow for formation of an aziridinium ion and subsequent alkylation. On this basis, cyclophosphamide would be expected to be a poor alkylating agent. It is also possible that differences in the uptake of phosphoramide mustard and cyclophosphamide as well as selective retention of the molecules within cells could contribute to different degrees of cytotoxicity.

We have compared the effect of cyclophosphamide and phosphoramide mustard on growth of leukemic cells in culture, and compared the degree of cross-linking of DNA in intact cells and nuclei when incubated with these drugs.

MATERIALS AND METHODS

Phosphoramide mustard with cyclohexylamine was a gift from the National Cancer Institute, Bethesda, Md, and [ring-4-^{14}C]cyclophosphamide monohydrate (specific activity, 11 millicuries/mmol) was obtained from New England Nuclear Corp, Boston, Mass. Cyclophosphamide monohydrate (minimum purity of 95% based on company specifications) was obtained from Koch Light Laboratories, Colnbrook, England. *Nor*-nitrogen mustard (β-chloroethylamine HCl) was obtained from ICN Pharmaceuticals, Plainview, NY. Cell culture materials were purchased from Grand Island Biological Co, NY. Ribonuclease, protease, calf thymus DNA, RNA, 8-hydroxyquinoline, and dextran 500 were obtained from Sigma Chemical Co, St. Louis, Mo; Darvan 1 was obtained from R. T. Vanderbilt Co, Inc, Norwalk, Conn; [methyl-^{3}H]thymidine (specific

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[2]Supported by IBM (Canada).

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activity, 5 curies/mmol) was obtained from Amersham/Searle, Arlington Heights, Ill; and carbowax 6000 was obtained from Analabs, North Haven, Conn.

Cell Source

Cell lines LS2 and LM4 were the strains of L1210 mouse leukemic cells described by Hillcoat (3) and are sensitive and resistant respectively to the drug methotrexate. The cells were maintained in suspension culture in RPMI 1640 medium containing 10% fetal calf serum by subculturing 2×10^5 cells/ml in 25-cm^2 (50-ml) tissue culture flasks. All cell concentrations were determined using a Model F Coulter Counter. When larger quantities of unlabeled cells were required, they were grown in BDF_1 mice by ip injection of 4×10^6 LM4 cells/mouse. Five days after inoculation, the mice were killed by ether inhalation, the cells were removed from the peritoneal cavity with saline solution and centrifuged at 600 × *g* for 10 minutes, and the pellet was collected.

Prelabeled Cells

LM4 cells, at 2–2.5 $\times 10^5$/ml, were grown for 24 hours under the usual conditions but with 5 μM unlabeled thymidine plus 0.1 microcurie/ml [methyl-^{3}H]thymidine.

Dose-Dependent Effect of Cyclophosphamide and Phosphoramide Mustard

Cells from LS2 and LM4 lines were resuspended at 2×10^5 cells/ml in medium containing 10% fetal calf serum and different concentrations of cyclophosphamide or phosphoramide mustard ranging from 1×10^{-3} to 1×10^{-7} M. The drugs were dissolved in medium immediately before use and were sterilized by filtration through a Millipore (0.22-μm) filter. The cells were continuously exposed to the drugs during growth at 37°C and were counted on the fourth day.

Effect of the Drugs After Various Times of Exposure

LM4 and LS2 cells were grown for 24 hours after subculturing. Phosphoramide mustard and cyclophosphamide were added to the cultures to give final concentrations of 1×10^{-4} and 1×10^{-3} M respectively. After 0, 1, 2, 3, and 4 hours' incubation of the cells with the drugs, the cells were centrifuged at 600 × *g* for 5 minutes, washed once with 5 ml of medium, again centrifuged at 600 × *g* for 5 minutes, and then resuspended in 10 ml of medium containing 10% fetal calf serum. Controls without drugs were treated similarly and all cells were counted on the fourth day.

Trypan Blue Dye Exclusion Test for Determination of Cell Viability

The test was based on the ability of living cells to exclude the dye, trypan blue. The method of Phillips (4) was followed.

Determination of the Purity of Cyclophosphamide

The purity of cyclophosphamide was determined by thin-layer and gas chromatography. In the first case, plates precoated with silica gel G-25 were developed in chloroform-ethanol (9:1), dried at room temperature, sprayed with 1% of 4-*p*-nitrobenzylpyridine in acetone, heated for 20 minutes at 120°C, and then sprayed with 3% KOH in methanol. Compounds with alkylating activity appeared as blue spots. In the second case, gas chromatographic analyses of cyclophosphamide and *nor*-nitrogen mustard were performed on a Varian 2100 gas chromatograph with a 6-foot column containing 3% SE30 on Chromosome G High Performance, 80/100 mesh with temperature program from 90°C to 250°C at 10°C/minute. The injector port and detector were kept at 300°C with a setting of 30 on the flow gauge (the flow gauge was adjusted to give 30 ml/minute flow rate of helium). Cyclophosphamide and *nor*-nitrogen mustard were derivatized with trifluoroacetic anhydride (5) before injection.

Determination of the Stability of Cyclophosphamide in Incubation Condition

Stability of cyclophosphamide in incubation medium during 4 hours of incubation at 37°C was determined by incubating 1×10^{-3} M cyclophosphamide in 1640 medium containing 0.04 microcurie of [ring-4-^{14}C]cyclophosphamide. Samples of 100 μl were taken before and after incubation and applied separately and together to plates for thin-layer chromatography. After drying, 0.5-cm bands were scraped from origin to solvent front and counted for radioactivity.

DNA Alkylated in Intact Cells Treated With Cyclophosphamide and Phosphoramide Mustard

The cells were grown in flasks for 24 hours with tritiated thymidine to label DNA. Average total amounts of DNA per flask were 39.8 ± 0.7 μg. At

that point, cyclophosphamide or phosphoramide mustard was added to give final concentrations of 1×10^{-3} or 1×10^{-4} M respectively. After 4 hours' incubation at 37°C, the suspension cultures were centrifuged at $600 \times g$ for 10 minutes and washed once with medium. Control cultures were prepared by adding medium to the culture in place of the drug solution. The cells could be stored frozen if necessary.

DNA Alkylated in Nuclei Treated With Cyclophosphamide, Phosphoramide Mustard, and *Nor*-Nitrogen Mustard

Nuclei from LM4 cells were prepared using the method of Rickwood et al (6). The nuclei were suspended in medium to give a concentration of 200 μg of DNA/ml. Carrier nuclei were prepared from LM4 cells grown in BDF_1 mice. A suspension of 2 ml of nuclei, in 10 ml of medium containing 10% fetal calf serum in a 25-ml Erlenmeyer flask, was treated with either 1×10^{-3} M cyclophosphamide, 1×10^{-4} M phosphoramide mustard, or *nor*-nitrogen mustard (1×10^{-5}, 5×10^{-5}, and 1×10^{-4} M). The latter concentrations were chosen to represent 1%, 5%, and 10% of the concentration of cyclophosphamide, since this covered the range of decomposition of cyclophosphamide observed under these conditions (described later). Controls were set up by adding medium instead of the drug solution. All of the flasks were incubated at 37°C for 4 hours with constant shaking and at the end of the incubation, 5 ml of cold medium and the carrier nuclei were added. The suspension was centrifuged at $1000 \times g$ for 10 minutes at 0°C and washed once with medium, and the pellet was collected for DNA isolation.

Isolation of DNA

DNA was isolated from intact cells and nuclei using the method of Walker (7). Two $\times 10^8$ cells were used per 10 ml of 5% (wt/vol) aqueous 4-amino sodium salicylate.

Additionally, the DNA preparation was kept at room temperature for approximately 20 minutes to allow for complete lysis of the cells. Three ml of ribonuclease solution of 1 mg/ml in 0.1 M NaCl-0.01 M acetate, pH 5.0, was then added per 100 ml of the cell lysate and incubated at 37°C for 20 minutes. Protease solution in 0.1 M NaCl-0.01 M acetate, pH 5.0, was then added to the lysate to give a final concentration of 0.5 mg/ml and was incubated at 37°C for 2 hours.

The resultant solution after phenol extraction was further deproteinized by shaking with an equal volume of $CHCl_3$-isoamyl alcohol (24:1), centrifuging at $1250 \times g$ for 20 minutes, collecting the upper phase, and then repeating the procedure. The upper phase was dialyzed against three changes of 0.1 M sodium acetate. To the dialysate, equal amounts of ethoxy ethanol were added and DNA was obtained on a glass rod by stirring. The isolated DNA was washed with 50% aqueous ethoxy ethanol and dissolved in 0.01 M phosphate buffer, pH 6.8, so that 1 ml of suspension was equivalent to 1.5×10^8 of original cells. If the DNA aqueous ethoxy ethanol mixture was too dilute to precipitate the DNA, it was concentrated on a rotary evaporator at 20°C with water suction. DNA concentration was estimated by the method of Burton (8), protein by the method of Lowry et al (9), and RNA by the orcinol procedure (10).

Estimation of Inter-Strand Cross-Linking of DNA

Denaturation of DNA results in strand separation except when cross-linking between strands is present. On rapid cooling and neutralization, the separated single strands do not renature. However, those strands held in proximity by cross-linking do renature under these conditions, reforming double-stranded DNA. Thus, the amount of double-stranded DNA present is a measure of the amount of inter-strand cross-linking present. To measure the amount of double-stranded DNA present, the polyethylene glycol system of Alberts (11) has been used. Double-stranded DNA partitions into the polyethylene glycol rich upper phase and single-stranded DNA partitions into the low phase. The isolated DNA was diluted in 10 mM phosphate buffer, pH 6.8, to give an approximate concentration of 400–500 μg/ml. The DNA was denatured by carefully mixing with an equal volume of 0.1 N NaOH, incubating at 37°C for 10 minutes, cooling, and adding an equal volume of ice-cold 0.1 M NaH_2PO_4. The cold solution was dialyzed at 4°C against 10 mM sodium phosphate, pH 6.8, with three changes of the buffer. The DNA samples were sonicated for 1 minute ($3 \times$ 20-second intervals) at 0°C, 30 KHz, and partitioned in the two-phase system of Alberts (11).

Partition of single- and double-stranded DNA was carried out by mixing 3 ml of the DNA solution, 1.2 ml of the lower phase and 1.3 ml of the upper phase, in 15-ml stoppered ice-chilled tubes. Because the lower phase was very viscous, it was measured and transferred with a graduated disposable syringe. The mixture was shaken vigorously and left overnight in the cold with occasional stirring on a Vortex mixer. Efficiency of partition was checked by using native double-stranded DNA. The amount of DNA extracted into the upper phase was measured by adding 250 μl of the sample to 10 ml of

Bray's scintillation solution and counting the sample in a Beckman scintillator; the unfractionated DNA solution was also counted in the same way. The percentage of DNA extracted was calculated from these values. Quench corrections were determined from a calibration curve.

RESULTS AND DISCUSSION

The effect of cyclophosphamide and phosphoramide mustard on the growth of LS2 and LM4 cells is shown in figure 1. The sensitivity of LM4 cells to cyclophosphamide and phosphoramide mustard was the same as that of LS2 cells. Phosphoramide mustard concentrations of 1×10^{-4} M produced 90% inhibition of growth while 50% inhibition was found between 2.5 and 5×10^{-5} M. Cyclophosphamide at 1×10^{-4} M showed no significant effect on the growth of LM4 and LS2 cells and at 1×10^{-3} M produced 15% growth inhibition in LS2 and 8% in LM4 cells. In agreement with previous reports by other investigators (12,13), these data demonstrate that phosphoramide mustard is 100 times more inhibitory than cyclophosphamide to cultured LM4 and LS2 cells.

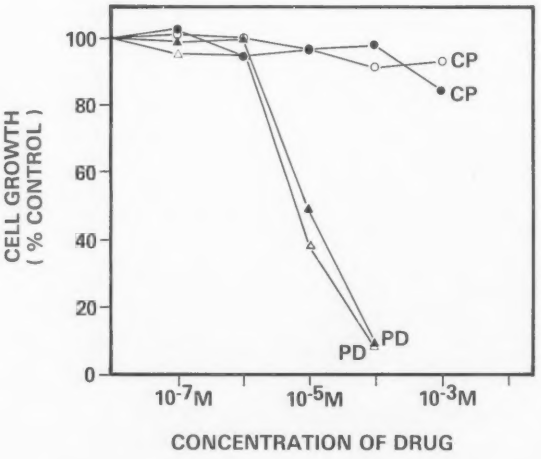

FIGURE 1.—Effect of varying concentration of cyclophosphamide and phosphoramide mustard on LM4 and LS2 cells. Cells were grown in the presence of varying concentrations of cyclophosphamide or phosphoramide mustard added at the time the cells were subcultured. Cells were allowed to grow until the control cells reached resting phase and were then counted. Each point is the average of 3 experiments and is expressed as a percentage of the control cells. ○ = LM4 treated with cyclophosphamide; ● = LS2 treated with cyclophosphamide; △ = LM4 treated with phosphoramide mustard; ▲ = LS2 treated with phosphoramide mustard.

Figure 2 shows that increasing the time of exposure of the cells to the same concentration of phosphoramide mustard gave increasing inhibition. Four hours of incubation with phosphoramide mustard results in approximately 80% growth inhibition, while no effect was observed with cyclophosphamide. To confirm that the growth inhibition observed was a measure of the cells killed, the trypan blue exclusion test was used to determine cell viability. We found that exposure of cells to phosphoramide mustard caused enlargement of the "live" cells, ie, cells which did not take up dye.

Thin-layer chromatography of the original sample of cyclophosphamide showed one blue spot, indicating it was the only alkylating agent present in detectable amounts.

On gas chromatography, trifluoroacetylated cyclophosphamide had a retention time of 17.42 minutes and trifluoroacetylated *nor*-nitrogen mustard had a retention time of 3.50 minutes. No trifluoroacetylated *nor*-nitrogen mustard was detected in the original sample of cyclophosphamide.

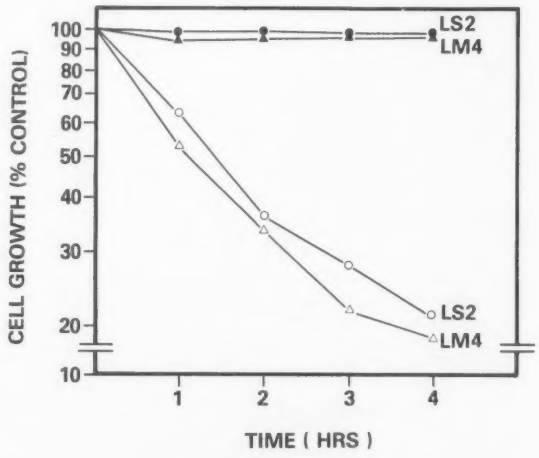

FIGURE 2.—Effect of cyclophosphamide and phosphoramide mustard at various time exposures. Cells were grown in the absence of drug for 24 hrs prior to addition of cyclophosphamide (1×10^{-3} M) and phosphoramide mustard (1×10^{-4} M). After 0, 1, 2, 3, and 4 hrs' incubation of LM4 and LS2 cells, the cultures were centrifuged at $600 \times g$ for 5 mins, washed with 5 ml of 1640 medium, and then resuspended in 10 ml of 1640 medium containing 10% fetal calf serum. Controls were set up without the drug. The cells were counted on the fourth day. Each experiment was performed 3 times. The results were expressed as the percentage of the control cells. A logarithmic scale has been used for vertical axis to render the curves more linear. △ = LM4 treated with phosphoramide mustard; ○ = LS2 treated with phosphoramide mustard; ▲ = LM4 treated with cyclophosphamide; ● = LS2 treated with cyclophosphamide.

The results of thin-layer chromatography of [ring-4-^{14}C]cyclophosphamide before and after incubation for 4 hours at 37°C in 1640 medium are shown in figure 3. All the recovered radiolabel was attributable to ^{14}C-cyclophosphamide. The recovery of ^{14}C-cyclophosphamide was 97.9% ± 5.6% (n = 8). These results show that at pH 7.4 there was minimal hydrolysis of cyclophosphamide to *nor*-nitrogen mustard in 4 hours, although Arnold and Klose (14) showed some hydrolysis at 37.5°C and pH 6.85 after 24 hours.

Effect of Cyclophosphamide, Phosphoramide Mustard, and *Nor*-Nitrogen Mustard on Intact Cells and Nuclei

The data in table 1 show that the biphasic partition method is suitable for separating double- from single-stranded DNA, in agreement with the previous reports of Alberts (11) and Walker (7). Thus, on the average, 79% of native DNA migrated to the upper layer of the biphasic system during the first extraction. When DNA solution was made alkaline and then neutralized, an average of 12.3% of the DNA was present in the upper layer at the first extraction. Most of this DNA is regarded as spontaneously renaturable DNA for whatever reason, and each subsequent extraction removed 1.2% of the input DNA into the upper layer. When LM4 cells grown for 24 hours were treated with 1 × 10^{-4} M phosphoramide mustard for 4 hours and their DNA was denatured, rapidly renatured, and extracted, 31% of the double-stranded DNA was extracted into the upper phase compared to 14.7% for the control (table 2). This difference is highly significant ($P < 0.001$) but the result for cyclophosphamide (15.6%) was not ($P > 0.10$). The approximately 15% of double-stranded DNA extracted into the upper phase in the control and cyclophosphamide-treated cells may represent some spontaneous renaturation of DNA or perhaps intra-strand double helix formed from the single-stranded DNA. Thus, the degree of cross-linking was the difference of these values, ie, 15%. Our background of spontaneously renaturing DNA was higher than the 6.5% of Walker (7) and Harrap and Gascoigne (15). The latter investigators found a net maximum value of 24% and 14% cross-linking in drug-resistant and drug-sensitive cell lines respectively using the alkylating agent, chlorambucil (15). Walker observed net values from 10.3% to 26.3% depending on the dose of mechlorethamine used (7). Thus, although our background is somewhat high, the degree of cross-linking compares with that reported in the literature. The degree of cross-linking, as determined by phase extraction, correlates with the behavior of DNA in alkaline sucrose gradients (15). Phase extraction alone, however, is an indirect measure of cross-linking, and will require confirmation by other methods of estimation. Nevertheless, our data are consistent with phosphoramide mustard cross-linking DNA in intact cells, further strengthening its role as the active metabolite of cyclophosphamide.

Table 3 shows that the amount of cross-linked DNA present after treatment of nuclei with cyclo-

TABLE 1.—Percentage of DNA extracted to the upper phase by the biphasic partition system*

Extraction No.	Denatured by alkali and reneutralized	Not denatured
1	12.3% ± 1.1%	79.9% ± 1.5%
2	1.3% ± 0.10%	
3	1.2% ± 0.10%	

*DNA was extracted as described in the text. Each sample was done twice. 2 experiments were done for all the samples.

TABLE 2.—Estimation of cross-linking of DNA from intact cells incubated with 1× 10^{-4} M phosphoramide mustard and 1 × 10^{-3} M cyclophosphamide*

	Control	Cyclophosphamide treated	Phosphoramide mustard treated
Concentration of DNA (μg/ml)	564 ± 26	556 ± 16	521 ± 13
Protein concentration (μg/ml)	21 ± 1	13 ± 0.6	16 ± 0.9
Concentration of RNA (μg/ml)	Negligible	Negligible	Negligible
% of DNA in upper phase	14.7% ± 0.5%†	15.6% ± 1.9%	30.9% ± 3.0%

*Each sample was done twice. 3 experiments were done for all the samples. Efficiency of extraction of the double-stranded DNA into the upper phase was determined for each sample. The % of DNA extracted into the upper phase was corrected to 100% of extraction efficiency. The extraction efficiency ranged from 70.5% ± 1.7% to 88.56% ± 2.04%.

†Mean ± SD.

phosphamide increased twofold, but after treatment with phosphoramide mustard it remained relatively the same, compared to the amount of cross-linking found after incubation of intact cells with the drugs. Although these drug concentrations were high, they are similar to the intracellular concentration achieved when intact cells were treated with 4×10^{-3} M (chart 2, ref 16) or 1×10^{-3} M cyclophosphamide (table 1, ref 17).

Table 4 shows that the amount of cross-linked DNA after treatment of nuclei with 1×10^{-5}, 5×10^{-5}, and 1×10^{-4} M *nor*-nitrogen mustard does not differ significantly from control values ($P > 0.10$). The results in table 4 show that the effect of

TABLE 3.—Estimation of cross-linking of DNA from nuclei incubation with 1×10^{-4} M phosphoramide mustard and 1×10^{-3} M cyclophosphamide*

	Control	Cyclophosphamide treated	Phosphoramide mustard treated
Concentration of DNA (μg/ml)	468 ± 16	402 ± 20	460 ± 14
Protein concentration (μg/ml)	Negligible	Negligible	Negligible
RNA concentration (μg/ml)	Negligible	Negligible	Negligible
% of DNA in upper phase	16.3 ± 3.3†	29.9% ± 3.5%	32.2% ± 4.2%

*Each sample was done twice. 3 experiments were done for all the samples. Efficiency of extraction of the double-stranded DNA into the upper phase was determined for each sample. The % of DNA extracted into the upper phase was corrected to 100% of extraction efficiency. The extraction ranged from 80.9% ± 1.9% to 95.0% ± 3.2%.

†Mean ± SD.

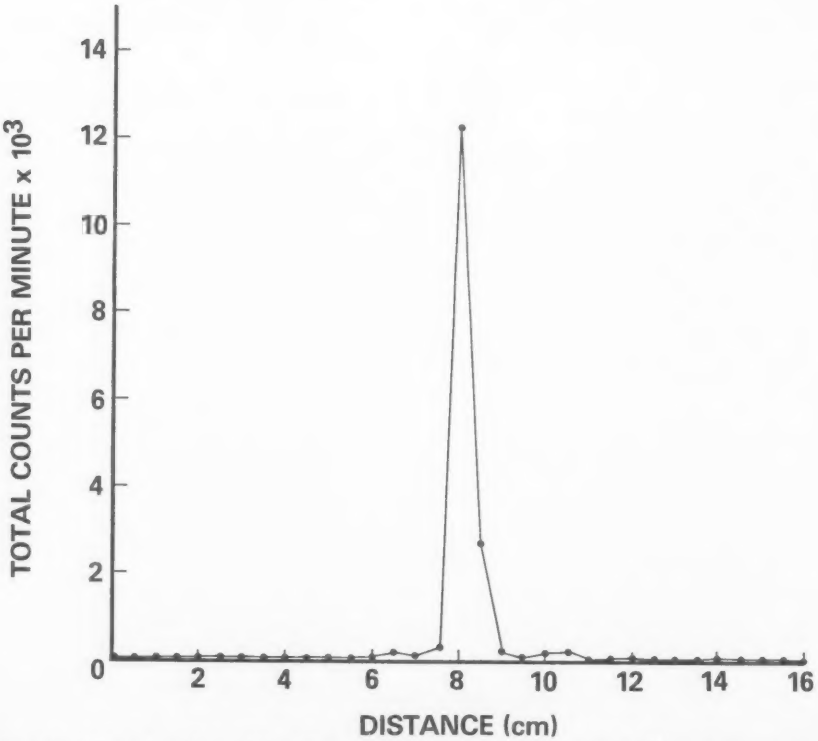

FIGURE 3.—Stability of cyclophosphamide in 1640 medium. Cyclophosphamide (1×10^{-3} M) in 1640 medium containing 0.04 microcurie of [ring-4-^{14}C]cyclophosphamide was incubated for 4 hrs at 37°C. After incubation, equal amounts of pre- and post-incubated samples were mixed, and then 100 μl of this mixture was spotted on 5 × 20-cm thin-layer plate (precoated with silica gel G-25). The solvent was chloroform-ethanol (9:1). The plate was dried at room temperature and a 0.5-cm band from the origin to the solvent front was scraped and counted for radioactivity. The result was expressed as total counts/min.

TABLE 4.—Estimation of cross-linking of DNA from nuclei incubation with cyclophosphamide, phosphoramide mustard, and *nor*-nitrogen mustard*

Drug used	Concentration of drug (M)	% of DNA in upper phase
Control	—	24.5 ± 5.0†
Cyclophosphamide	1×10^{-3}	37.2 ± 7.4
Phosphoramide mustard	1×10^{-4}	40.7 ± 9.0
Nor-nitrogen mustard	1×10^{-5}	27.9 ± 6.8
Nor-nitrogen mustard	5×10^{-5}	26.4 ± 9.1
Nor-nitrogen mustard	1×10^{-4}	25.9 ± 5.7

*The DNA concentration used for all the samples was approximately 500 μg/ml. Negligible amounts of protein and RNA were found. Each sample was done twice. 7 experiments were done for all the samples. Efficiency of extraction of the double-stranded DNA into the upper phase was determined for each sample. The % of DNA extracted into the upper phase was corrected to 100% of extraction efficiency. The extraction ranged from 78.9% ± 4.1% to 88.1% ± 3.2%.

†Mean ± SD.

cyclophosphamide on the nuclei is not caused by *nor*-nitrogen mustard produced by hydrolysis of cyclophosphamide. These experiments were carried out with a fresh set of reagents and at a different time from those experiments reported in tables 1–3. This may account for the higher control value obtained in these seven experiments. However, the net increase in the percentage of double-stranded DNA (presumably cross-linked) with phosphoramide mustard was 16.2% with intact cells (table 2) and 15.9% and 16.2% with nuclei (tables 3 and 4 respectively).

There is no evidence that nuclei can activate cyclophosphamide. High levels of this drug occur in the cytoplasm of cells (*see* above), so it is not clear at this time why this drug alkylates DNA in isolated nuclei but not in intact cells.

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Disposition of Chlorozotocin in Rats and Dogs [1]

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SUMMARY

Chlorozotocin is a new chloroethyl nitrosourea with antineoplastic activity. Its disposition in rats and dogs was investigated by measurement of total ^{14}C in biologic material after administration of chlorozotocin-[glucose-1-^{14}C] and chlorozotocin-[ethyl-2-^{14}C]. After iv administration to rats, the renal clearance of the glucose-^{14}C was rapid with 75% of the dose appearing in urine within 6 hours and only 4% in bile. In contrast, 37% of the ethyl-^{14}C was excreted in urine within 6 hours and 40% in bile. Chromatography of the bile showed one major and at least two minor products. There appears to be an enterohepatic circulation of the ethyl-^{14}C. Urinary excretion of both labels in the dog was similar to that in the rat, but slightly slower. The levels of ^{14}C in plasma declined in a biphasic manner. The half-life for the initial distribution phase (14 minutes) and that for the unbound radioactivity for the second phase (146 minutes) were independent of dose, position of label, and animal species. Binding markedly reduced the rate of decline of total radioactivity in plasma after the distribution phase and accounts for differences in the half-lives for the two labeled species. Tissue distribution studies in rats at 6 hours and dogs at 24 hours showed significantly higher concentrations of ethyl-^{14}C than glucose-^{14}C in the liver and kidney. Levels of ethyl-^{14}C initially were also significantly higher in the cerebrospinal fluid giving further support for the view that cleavage of the chlorozotocin molecule occurs between its glucose and chloroethyl moieties.

[Cancer Treat Rep 62:31–44, 1978]

Chlorozotocin (fig 1) is a new water-soluble nitrosourea synthesized by Johnston et al (1). It is the 2-chloroethyl analog of the antineoplastic antibiotic streptozotocin. Chlorozotocin exhibits significant antitumor action against L1210 leukemia cells implanted ip in mice, but importantly, like streptozotocin, it appears to be strikingly less toxic to bone marrow function than BCNU (2,3). Although streptozotocin is known to be diabetogenic in rats and dogs (4), chlorozotocin exhibited no such activity in mice (5). Assuming that chlorozotocin proves to be a nondiabetogenic antitumor agent with low myelosuppressive activity in man the drug would indeed represent a significant advance in the therapy of neoplastic disease.

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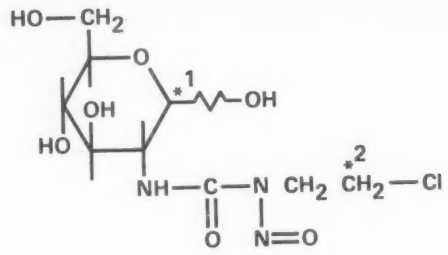
The present studies with chlorozotocin were undertaken to investigate its tissue distribution as well as its rate of transformation and excretion. Measurement and identification of chlorozotocin and its products is complicated by the aqueous solubility of the drug and its chemical instability. Therefore, only the total radioactivity in tissues was measured after administration of ethyl-^{14}C- and glucose-^{14}C-chlorozotocin to rats and dogs.

METHODS AND MATERIALS

Chlorozotocin (purity > 99%) was obtained from the DTP, DCT, NCI (Bethesda, Md). Chlorozotocin-[ethyl-2-^{14}C] (16.1 millicuries/mmol) and chlorozotocin-[glucose-1-^{14}C] (17.1 millicuries/mmol) were synthesized by Monsanto Research Corp (Dayton, Ohio). Their radiopurity was shown to be 95%–96% as determined by thin-layer chromatography (TLC) on silica gel (Baker-flex 1B) using chloroform:methanol (3:1, vol/vol) and by 2-dimensional TLC using acetone as the solvent system. The drug was dis-



*Position of label 1 Glucose - 1 - ^{14}C

2 Ethyl - 2 - ^{14}C

FIGURE 1.—Structure of chlorozotocin (NSC-178248; 2-[3-(2-chloroethyl)-3-nitrosoureido]-2-deoxy-D-glucopyranose).

solved in 0.005 M citrate, pH 5, immediately before administration to animals.

Male Sprague-Dawley rats (Taconic Farms, Inc, Germantown, NY) and female foxhounds (NIH breeding colony, Poolesville, Md) were used in these studies. Rats, weighing 220–260 g, received [^{14}C]-chlorozotocin (2 microcuries, 10 mg/kg ip) and were then housed in glass metabolic cages, with access to food and water, for 96 hours. Urine and feces were collected separately by the anal cup technique (6). Plasma levels and biliary excretion of chlorozotocin were measured following iv administration. The rats were anesthetized with pentobarbital (40 mg/kg) and cannulas were inserted into the common bile duct and into the aorta above the bifurcation of the iliacs. [^{14}C]-chlorozotocin (5, 10, or 20 mg/kg) was given via the femoral vein. The animals were then placed in restraining cages and allowed to recover from anesthesia. Bile samples were collected continuously in vials on ice, and blood samples were withdrawn at timed intervals into a heparinized syringe. After 6 hours, the rats were killed with an overdose of pentobarbital, and tissues were immediately excised, weighed, and frozen. Urine was collected throughout the experiment and, at 6 hours, the urine in the bladder was removed.

In a separate experiment, noncannulated rats were anesthetized 6 hours after receiving [^{14}C]-chlorozotocin (10 mg/kg) iv. Blood samples were withdrawn from the aorta and the animals were then killed with additional pentobarbital. Both femurs were removed. Bone marrow cells were expressed from the right femur with physiologic saline and a cell count was determined on a Model B Coulter counter. The left femur was cracked open and the bone marrow was removed and weighed.

Female foxhounds, weighing 14–17 kg, were anesthetized with pentobarbital (30 mg/kg) and were maintained in this state throughout the experiment with additional pentobarbital. [^{14}C]-chlorozotocin (50 microcuries, 0.75 mg/kg) was administered to the dogs via a catheter inserted in the femoral vein. Blood samples were withdrawn from the femoral artery by means of an indwelling catheter and immediately transferred to heparinized tubes chilled on crushed ice and centrifuged. Cerebrospinal fluid (CSF) was obtained through PE-100 tubing attached to a 19-gauge needle inserted into the cisterna magna. Urine samples were obtained by continuous drainage from the bladder using an indwelling Foley catheter and were collected in flasks on ice. Six hours after the administration of chlorozotocin, the catheters were removed, 3 ml of Combiotic (Pfizer and Co, Inc, New York, NY) was administered im, and the dogs were placed in individual cages to recover from anesthesia and to allow collection of urine for an additional 18 hours. Blood was taken from the re-anesthetized dog after 24 hours and urine was removed from the bladder. In addition, a 24-hour CSF sample was obtained by reinserting a 19-gauge needle attached to a syringe into the cisterna magna. Subsequently the dog was killed with an overdose of pentobarbital. A section of the liver was removed for the subcellular distribution study and then the remaining tissues were excised, blotted, and weighed.

Preparation of samples.—The radioactivity in aliquots of drug solution, bile, and urine was measured by liquid scintillation counting with 15 ml of phosphor (5 ml of Triton X-100 with 150 mg of 2,5-diphenyloxazole and 1.9 mg of 1,4-bis-[2-(5-phenyloxazolyl)] benzene dissolved in 10 ml of toluene). Aliquots of plasma (0.25 ml for dogs, 0.05 ml for rats) were incubated at 45°C for 45 minutes with 1 ml of NCS tissue solubilizer (Amersham Searle Corp, Arlington Heights, Ill) before addition of phosphor. Bound radioactivity was determined by precipitating the protein from aliquots of plasma (0.5 ml for dogs, 0.05 ml for rats) with 1 ml of 10% trichloroacetic acid. The precipitate was washed twice with 1-ml volumes of 10% trichloroacetic acid and then dissolved in 2 ml of NCS. Samples of the dog tissues were homogenized in 4 volumes of water and then 2-ml aliquots (1 ml for liver, 0.2 ml for spleen) were solubilized by incubation for 24 hours at 45°C with 2 ml of NCS. For the rats, the whole tissues and the residual carcass were homogenized separately in a known volume of water. Bone marrow samples, in the form of a cell suspension or as solid material, were digested directly with NCS. All NCS-digested samples were neutralized with glacial acetic acid (0.1 ml/2 ml of NCS) before addition of phosphor.

Radioactivity was measured by liquid scintillation counting, and all data were corrected for counting efficiency by external standardization.

Half-life determinations.—Groups of four animals were administered chlorozotocin-[glucose-1-^{14}C] or chlorozotocin-[ethyl-2-^{14}C] and levels of radioactivity in plasma and CSF were measured at intervals thereafter. Analyses of differences between the two groups of animals with regard to mean plasma and CSF ^{14}C levels were made using the Student's t test. The mean plasma levels of radioactivity were fitted to the equation, $y = a \exp(bt)$, where y = plasma concentration (μg of chlorozotocin equivalents/ml) at time t (min), a = y intercept, and b = slope of semilogarithmic plot of the disappearance of radioactivity in plasma by a nonlinear least-squares program on a Wang 600 computer. The decline of radioactivity in plasma was assumed to be biexponential. The values for the first or rapid phase were obtained from the data by extrapolating a line through the second or slower phase points to the y intercept and subtracting the values of the slower phase from the faster phase portion of the curve. The half-lives ($t_{\frac{1}{2}}$) were then determined using the relationship $t_{\frac{1}{2}}=0.693/b$.

Thin-layer chromatography.—Urine from dogs and bile from rats were collected after administration of [^{14}C]-chlorozotocin, and aliquots were applied to silica gel TLC sheets (Baker-flex 1B) which were developed in butanol:acetic acid:water (3:1:1, vol/vol). Radioactive spots on the chromatograms were located by autoradiography using Kodak No-Screen NS54T X-ray film. The spots were cut from the chromatograms and the radioactivity was determined by liquid scintillation counting with 10 ml of Econofluor (New England Nuclear Corp, Boston, Mass).

Preparation of cell fractions.—Liver samples (30–40 g) were chilled immediately on crushed ice and were homogenized with 3 ml of isotonic sucrose (0.25 M) per gram of tissue using a Polytron homogenizer (Brinkman Instrument Co, Westbury, NY). Cell fractions were prepared by differential centrifugation (7). The nuclei and cell debris were sedimented by centrifugation of the whole homogenate at 600 g_{avg} for 30 minutes. The supernatant was then recentrifuged for 30 minutes at 10,000 g_{avg} to sediment mitochondria. To isolate microsomes, the 10,000 g supernatant fraction was recentrifuged at 105,000 g_{avg} for 60 minutes. For the measurement of radioactivity, 2-ml aliquots of the homogenate and supernatant fractions were transferred to liquid scintillation counting vials and incubated overnight at 45°C with 2 ml of NCS. The samples were cooled to room temperature and 0.1 ml of glacial acetic acid and 15 ml of phosphor were added.

RESULTS

Excretion of radioactivity in rats and dogs.—The cumulative excretion of radioactivity in the urine and feces of rats after administration of chlorozotocin-[glucose-1-^{14}C] or chlorozotocin-[ethyl-2-^{14}C] (10 mg/kg ip) is shown in table 1. Six hours after dosing, a significantly ($P < 0.005$) greater amount of radioactivity had been excreted in the urine of rats treated with glucose-^{14}C- than with ethyl-^{14}C-chlorozotocin. However, by 24 hours, 77.1% of the administered ^{14}C was recovered in urine regardless of the position of the label. Although only a relatively

TABLE 1.—Excretion of radioactivity by the rat after ip administration of [^{14}C]-chlorozotocin*

	Time (hrs)	Cumulative excretion (%) of—	
		[Ethyl-2-^{14}C]	[Glucose-1-^{14}C]
Urine	6	39.8 ± 6.8†	70.6 ± 8.0
	24	77.1 ± 2.6	77.1 ± 5.1
	48	79.0 ± 2.7	77.8 ± 5.0
	72	79.6 ± 2.7	78.2 ± 4.9
	96	80.0 ± 2.7	78.4 ± 4.9
Feces	24	1.7 ± 0.7†	5.9 ± 0.5
	48	2.0 ± 0.8†	7.2 ± 1.1
	72	2.0 ± 0.8†	7.4 ± 1.1
	96	2.1 ± 0.8†	7.5 ± 1.1
Carcass		3.8 ± 0.2†	2.6 ± 0.2
Total recovery		85.9 ± 3.1	88.5 ± 3.8

*[^{14}C]-chlorozotocin (10 mg/kg ip) was administered to male Sprague-Dawley rats. Radioactivity in urine, feces, and carcass was determined as described in the Methods section. Each value represents the mean ± SD from 4 rats.

†Values of the ethyl-^{14}C are significantly different ($P < 0.005$) from those of the glucose-^{14}C.

TABLE 2.—Tissue distribution of radioactivity in rats*

	Distribution (μg/g)† of—	
	[Ethyl-2-^{14}C]	[Glucose-1-^{14}C]
Kidneys	12.95 ± 2.15‡	6.99 ± 1.68
Liver	7.09 ± 1.57§	4.26 ± 1.13
Spleen	4.99 ± 1.01§	11.24 ± 3.58
Pancreas	3.75 ± 0.50	4.67 ± 1.35
Small intestine	2.78 ± 0.47‡	6.98 ± 0.86
Lung	2.44 ± 0.26	2.89 ± 0.49
Plasma	1.89 ± 0.21¶	1.25 ± 0.40
Large intestine	1.67 ± 0.53	1.96 ± 0.70
Heart	1.41 ± 0.12§	2.08 ± 0.27
Stomach	0.61 ± 0.16	0.87 ± 0.22
Muscle	0.54 ± 0.20	0.74 ± 0.06
Brain	0.52 ± 0.10	0.62 ± 0.08
	% of dose recovered	
Urine**	37.4 ± 3.2‡	74.5 ± 8.2
Bile**	40.1 ± 4.3‡	4.3 ± 1.4
Tissues	6.2 ± 0.9	5.5 ± 1.1
Carcass	6.3 ± 0.6	10.3 ± 5.8
Total recovery	90.1 ± 8.4	94.0 ± 1.3

*Radioactivity in tissues 6 hrs after administration of ethyl-^{14}C- and glucose-^{14}C-chlorozotocin to rats (10 mg/kg iv) was determined as described in the Methods section. Each value is the mean obtained from 5 animals ± SD.

†μg of chlorozotocin equivalents/g of wet tissue, determined from the specific activity of administered chlorozotocin. Concentrations of ethyl- and glucose-^{14}C are significantly different.

‡$P < 0.005$.

§$P < 0.05$.

¶μg/ml.

**Cumulative excretion.

small amount of radioactivity was eliminated in the feces, that from rats receiving the glucose-^{14}C label was significantly greater. Six hours after iv dosing, a similar pattern for renal clearance was noted, with 74.5% ± 8.2% of the administered glucose-^{14}C label and 37.4% ± 3.2% of the ethyl-^{14}C label recovered in the urine (table 2). A difference in the 6-hour urinary excretion of the two labels was also observed in dogs given the highest nontoxic dose of [^{14}C]-chlorozotocin (0.75 mg/kg iv). The percentages of glucose-^{14}C and ethyl-^{14}C eliminated were 64.3% ± 2.1% and 30.0% ± 7.4%, respectively ($P < 0.005$). However, unlike the rat, the cumulative urinary excretion of the glucose-^{14}C (71.7% ± 2.4%) and the ethyl-^{14}C (55.9% ± 5.6%) also differed markedly after 24 hours ($P < 0.05$, table 3).

Disappearance of chlorozotocin from plasma.—For at least 6 hours after the iv administration of chlorozotocin (5, 10, or 20 mg/kg) to rats, the levels of radioactivity in plasma were proportional to the dose of the drug. The levels of radioactivity, both total and unbound, in plasma after the administration of glucose- and ethyl-labeled chlorozotocin (10 mg/kg iv) are shown in figure 2. In each case the levels of radioactivity in plasma declined in a biphasic manner. During the first hour after dosing, the disappearance of the total and unbound radioactivity from the plasma was rapid ($t_{\frac{1}{2}}$ = 14 minutes) and independent of both the position of the label and the dose of the drug (table 4). During the second or slower phase, lasting from 1 to 6 hours, the total radioactivity derived from glucose-^{14}C-chlorozotocin disappeared from the plasma more rapidly than that derived from the ethyl-^{14}C-chlorozotocin. Moreover, clearance of the unbound radioactivity was more rapid than that of the total radioactivity, and both unbound labeled species declined at the same rate. These data illustrate the influence that binding has on the rate of decline of radioactivity in plasma. The binding of the ethyl-^{14}C in plasma was significantly greater than that of the glucose-^{14}C. For example, after 3 hours, 65.5% ± 5.2% of the ethyl-^{14}C and only 45.8% ± 4.2% of the glucose-^{14}C were bound ($P < 0.005$). Since radioactivity bound to plasma proteins was measured after the precipitation of the proteins with trichloroacetic acid followed by two washings of the precipitate with additional acid, it is presumed not only that the nature of the binding is irreversible, but also that it is responsible for the long biologic $t_{\frac{1}{2}}$ of the total

TABLE 3.—Tissue distribution of radioactivity in dogs*

	Distribution (μg/g)† of—	
	[Ethyl-2-^{14}C]	[Glucose-1-^{14}C]
Liver	3.32 ± 0.46‡	0.71 ± 0.16
Kidneys	1.37 ± 0.35§	0.62 ± 0.14
Bladder	0.70 ± 0.11	0.41 ± 0.17
Large intestine	0.64 ± 0.24	1.21 ± 0.24
Pancreas	0.33 ± 0.13	0.22 ± 0.06
Small intestine	0.24 ± 0.07	0.30 ± 0.05
Lung	0.19 ± 0.03	0.22 ± 0.04
Plasma	0.16 ± 0.02§,¶	0.66 ± 0.16
Spleen	0.16 ± 0.08	0.34 ± 0.08
Ovary, uterus, vagina	0.16 ± 0.05	0.16 ± 0.04
Stomach	0.12 ± 0.03	0.15 ± 0.01
Heart	0.12 ± 0.03§	0.19 ± 0.02
Brain	0.07 ± 0.01	0.10 ± 0.01
Muscle	0.07 ± 0.01	0.07 ± 0.01
Omentum	**	0.05 ± 0.01
	% of dose recovered	
Urine (cumulative)	55.9 ± 5.6§	71.7 ± 2.4
Bile	3.5 ± 1.5	1.2 ± 0.2
Tissues	21.5 ± 3.5††	15.5 ± 3.0
Total recovery	80.5 ± 7.4	88.4 ± 4.9

*Radioactivity in tissues 24 hrs after administration of ethyl-^{14}C- and glucose-^{14}C-chlorozotocin to dogs (0.75 mg/kg iv) was determined as described in the Methods section. Each value is the mean obtained from 3 animals ± SD.

†μg/g of wet tissue.

§$P < 0.05$.

¶μg/ml.

**Not done.

††Includes plasma and muscle (46% body weight [ref 8]).

TABLE 4.—Parameters of the decline of radioactivity in plasma of rats after [^{14}C]-chlorozotocin*

Dose (mg/kg)	^{14}C measured	[Ethyl-2-^{14}C] y intercept (a) (μg/ml)	[Ethyl-2-^{14}C] $t_{\frac{1}{2}}$ (mins)	[Glucose-1-^{14}C] y intercept	[Glucose-1-^{14}C] $t_{\frac{1}{2}}$ (mins)
			First phase		
5	Total	10.9	14	†	
10	Total	18.5	14	27.0	14
	Unbound	18.5	14	25.4	15
20	Total	37.2	14	50.9	16
			Second phase		
5	Total	1.83	587	†	
10	Total	2.62	723	3.06	273
	Unbound	1.81	147	2.44	146
20	Total	6.12	619	6.58	273

*Plasma levels of chlorozotocin were fitted to the equation $y = a \exp(bt)$ as described in the Methods section; plasma concentration used for each point was the average value obtained from 4 rats. Concentrations are expressed as μg of chlorozotocin equivalents/ml of plasma.

†Not done.

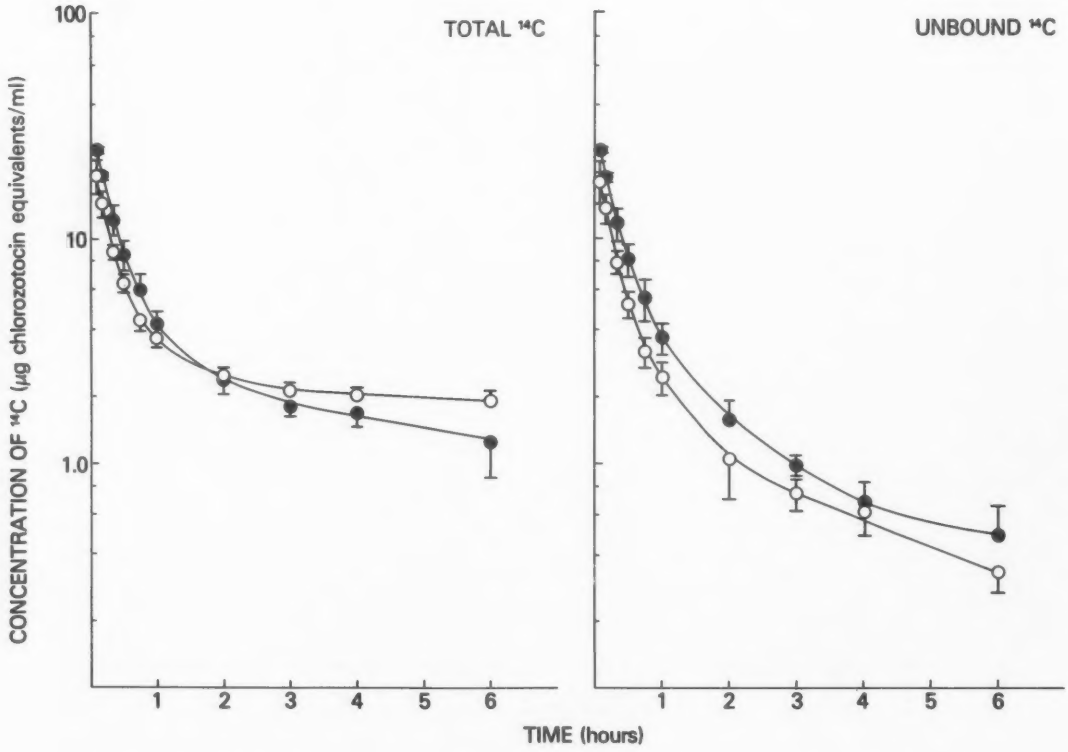


FIGURE 2.—Levels of radioactivity in plasma of rats. Chlorozotocin-[ethyl-2-^{14}C] (○——○) or chlorozotocin-[glucose-1-^{14}C] (●——●) was administered to rats (10 mg/kg iv). Each point is the mean value obtained from 4 rats ± SD. Where both positive and negative SDs are shown the values for the 2 labeled species are significantly different ($P < 0.05$). Concentrations are expressed as μg of chlorozotocin equivalents/ml.

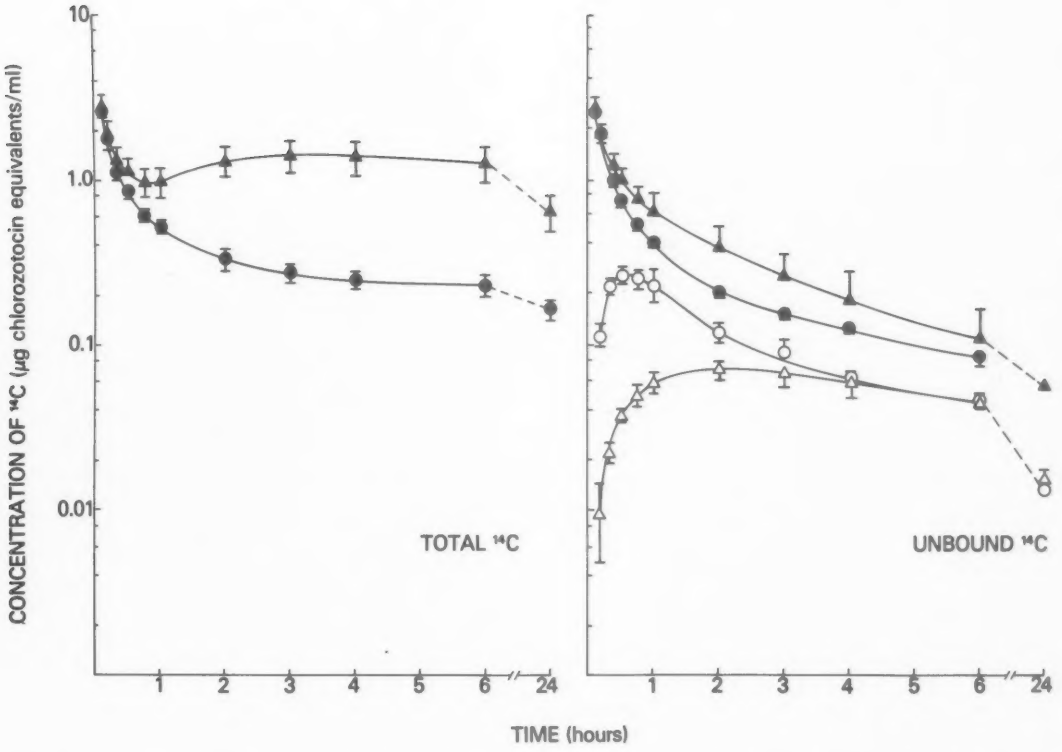


FIGURE 3.—Levels of radioactivity in plasma and CSF of dogs. [¹⁴C]-chlorozotocin was administered to anesthetized dogs (0.75 mg/kg iv). Levels of radioactivity were determined in plasma (●——● = ethyl-¹⁴C;▲——▲ = glucose-¹⁴C) and in CSF (○——○ = ethyl-¹⁴C; △——△ = glucose-¹⁴C). Each point is the mean value obtained from 3 dogs ± SD. Both positive and negative SDs are shown only where the difference between the ethyl and glucose labels is significant ($P < 0.05$). Concentrations are expressed as μg of chlorozotocin equivalents/ml.

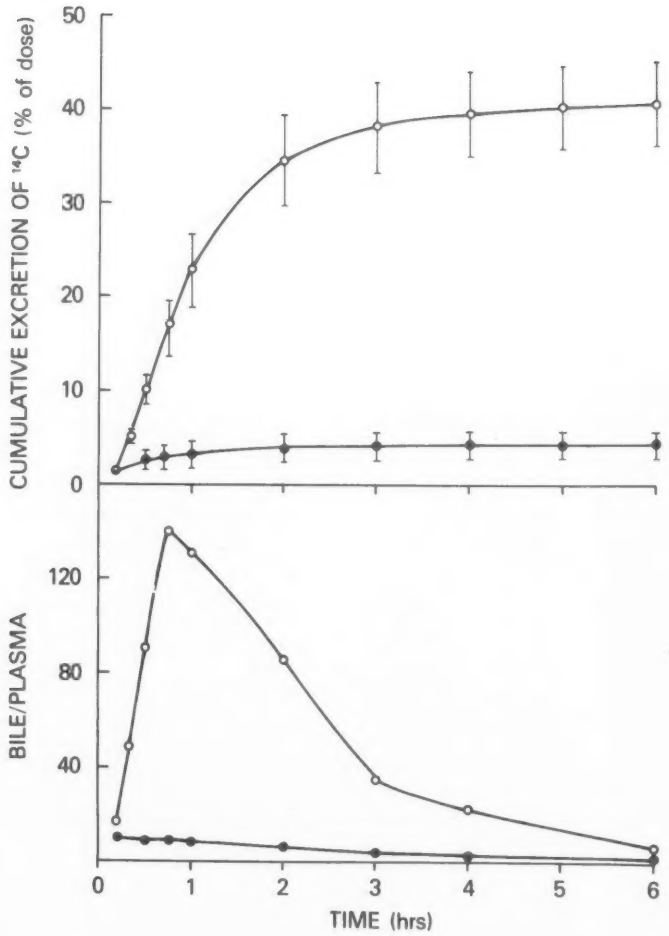


FIGURE 4.—Biliary excretion and bile:plasma ratios of radioactivity in rats. Chlorozotocin-[ethyl-2-^{14}C] (○———○) and chlorozotocin-[glucose-1-^{14}C] (●———●) were administered to rats (10 mg/kg iv). Each point is the mean value obtained from 4 rats. SDs are included for the cumulative excretion of radioactivity in bile.

TABLE 5.—Parameters of the decline of radioactivity in plasma of dogs after [^{14}C]-chlorozotocin*

Position of label	^{14}C measured	y intercept (a) (μg/ml)	$t_{\frac{1}{2}}$ (mins)
	First phase		
Glucose	Unbound	2.36	10
Ethyl	Unbound	2.59	11
Ethyl	Total	2.26	15
	Second phase		
Glucose	Unbound	0.82	121
Ethyl	Unbound	0.43	141
Ethyl	Total	0.37	495

*Plasma levels of chlorozotocin were fitted to the equation $y = a \exp(bt)$ as described in the Methods section; concentration used for each point was the average value obtained from 3 dogs. Concentrations are expressed as μg of chlorozotocin equivalents/ml of plasma.

radioactivity (587–723 minutes, table 4) observed with the ethyl-^{14}C compared to the $t_{\frac{1}{2}}$ of 273 minutes observed with the glucose-^{14}C. The profile of the curves for the disappearance of both total and unbound ethyl-^{14}C-chlorozotocin from the plasma of dogs, as well as that for the unbound glucose-^{14}C species, was generally similar to that observed with rats (fig 3). Moreover, there was no appreciable difference between dogs and rats in the biologic $t_{\frac{1}{2}}$ for these species (table 5). However, in dogs, after an initial rapid decline, the levels of total radioactivity in plasma following treatment with glucose-^{14}C-chlorozotocin increased and remained elevated for at least 4 hours (fig 3).

Disposition of radioactivity in brain and CSF.—Radioactivity was detected in the CSF of dogs after the administration of both the ethyl-^{14}C- and glucose-^{14}C-labeled drug (fig 3). The radioactivity from the ethyl-^{14}C species not only rose more rapidly than that from the glucose-^{14}C but also attained significantly higher concentrations in the CSF. The highest concentration of ethyl-^{14}C was measured 30 minutes after administration of the drug, thereafter declining over several hours to levels which were not significantly different from the maximum concentration of radioactivity from glucose-^{14}C. A steady state ratio of 2 to 1 was reached between levels of unbound ethyl-^{14}C in the plasma and levels of ethyl-^{14}C in the CSF by 45 minutes.

To examine more closely its distribution between CSF and brain tissue, ethyl-^{14}C-chlorozotocin was administered iv to two additional dogs. Samples of CSF were taken at 5-minute intervals for a period of 30 minutes at which time the animals were killed. The brains were immediately removed and samples of cerebrum, cerebellum, and medulla (including brain cord) were homogenized and their content of radioactivity was measured as described in the Methods section. Again the highest concentration of ethyl-^{14}C in the CSF was observed after 30 minutes and the radioactivity was uniformly distributed throughout the brain. For example, concentrations of 0.266 μg of chlorozotocin equivalents/ml were measured in the CSF of both dogs and 0.235–0.259, 0.211–0.242, and 0.233–0.242 μg of chlorozotocin equivalents/g in the cerebrum, cerebellum, and medulla, respectively. After 24 hours, however, the concentrations of ethyl-^{14}C in brain tissue were five times those in the CSF (fig 3 and table 3).

Biliary excretion of radioactivity in rats.—The amount of radioactivity recovered in the bile of rats treated with [^{14}C]-chlorozotocin was strongly influenced by the position of the label on the chlorozotocin molecule (fig 4). Only 4% of the dose of chlorozotocin-[glucose-1-^{14}C] (10 and 20 mg/kg iv) was excreted in bile within 6 hours. In contrast, 40% of the dose of the ethyl-^{14}C drug (10 mg/kg) was recovered in bile, the excretion being essentially complete within 3 hours. These findings provide evidence in support of the view that an extensive cleavage of the chlorozotocin molecule occurs at some point between its glucose and chloroethyl moieties. It is noteworthy that although a large proportion of the ethyl-^{14}C dose was excreted in the bile of cannulated rats, only a small proportion appeared in the feces of rats which had no biliary cannula (table 1). These observations are thus consistent with the view that an enterohepatic circulation of a species carrying the chloroethyl moiety occurs.

The rate of biliary excretion of the ethyl-^{14}C species was most rapid within the first hour and thereafter declined rapidly over the next 5 hours. As the dose was increased, the percentage of administered radioactivity excreted in bile tended to decrease. The cumulative percentages recovered from bile in 6 hours were 46.0% ± 4.3%, 40.2% ± 4.3%, and 34.6% ± 5.2% for doses of 5, 10, and 20 mg/kg, respectively.

Figure 4 also shows the bile:plasma ratios of

TABLE 6.—Subcellular distribution of radioactivity in liver*

	Distribution† of—			
	[Ethyl-2-^{14}C]		[Glucose-1-^{14}C]	
	3.69	3.46	0.89	0.57
	% drug in fraction			
Nuclei	12	18	24	26
Mitochondria	1	0	18	14
Microsomes	6	2	16	13
Soluble	81	80	42	47

*[^{14}C]-chlorozotocin was administered to anesthetized dogs (0.75 mg/kg iv). After 24 hrs, the dogs were killed and liver samples (30–40 g) were homogenized with 3 vols of 0.25 M sucrose. Aliquots of the mixtures were fractionated by differential centrifugation as described in the Methods section. Results are the average obtained from 2 homogenates.

† μg of chlorozotocin equivalents/g of wet tissue.

radioactivity at intervals after the administration of ethyl-^{14}C- and glucose-^{14}C-chlorozotocin. In view of the extensive biliary excretion of radioactivity following treatment with ethyl-^{14}C-chlorozotocin, it was not surprising to find large bile:plasma ratios. All of the ratios that were measured from 10 minutes to 5 hours after dosing with ethyl-^{14}C-labeled drug were > 10 and the highest ratio (130) was observed at 45 minutes. These findings suggest that the species containing the ethyl-^{14}C moiety is actively transported into bile. In contrast, bile:plasma ratios of radioactivity remained close to unity during the 6 hours following administration of glucose-^{14}C-chlorozotocin.

Distribution of radioactivity in tissues of rats and dogs.—The marked difference between the two labeled species of chlorozotocin with regard to mode of excretion in rats during the first 6 hours after iv administration of the drug suggested that differences might exist in the tissue distribution of the remaining radioactive material. Six hours after administration of the ethyl-^{14}C- or glucose-^{14}C-labeled drug to rats with biliary cannulas, concentrations of radioactivity greater than those in plasma were found in a number of tissues, notably kidney and liver (table 2). In these tissues the concentration of the ethyl-^{14}C was almost twice that of the glucose-^{14}C. The concentrations of the glucose-^{14}C, however, were significantly greater in spleen, small intestine, and heart. Concentrations in the tissues were approximately proportional to the dose of the drug. In a separate study, the levels of radioactivity in plasma and bone marrow were measured in noncannulated rats 6 hours following administration of the ethyl- or glucose-labeled chlorozotocin (10 mg/kg iv). Concentrations of the glucose-^{14}C species in plasma and bone marrow were 1.33 ± 0.10 μg of chlorozotocin equivalents/ml and 4.41 ± 0.40 μg/g (1.58 ± 0.33 ng/10^7 cells), respectively. Concentrations of the ethyl-^{14}C species were slightly higher with 2.68 ± 0.64 μg/ml of plasma and 5.86 ± 2.11 μg/g of bone marrow (3.91 ± 0.35 ng/10^7 cells). At this time, $80.6\% \pm 1.8\%$ and $52.1\% \pm 9.5\%$ of the glucose- and ethyl-^{14}C, respectively, had been excreted in the urine.

Twenty-four hours after administration of [^{14}C]-chlorozotocin (0.75 mg/kg iv) to dogs, considerably less radioactivity was recovered in urine of animals given ethyl-^{14}C-chlorozotocin compared to those given the glucose-^{14}C-labeled drug (table 3). Indeed, the measurement of the radioactivity in the tissues confirmed that significantly more ethyl-^{14}C than glucose-^{14}C remained in the animals: $15.9\% \pm 3.8\%$ and $6.8\% \pm 1.3\%$, respectively, excluding that in the carcass ($P < 0.05$). The highest concentration of ethyl-^{14}C was measured in the liver and was about fivefold higher than that of the glucose-^{14}C. As was observed in rats after 6 hours, the concentration of ethyl-^{14}C was approximately twice that of glucose-^{14}C in the kidney and was significantly lower than the glucose-^{14}C in spleen and heart.

Subcellular distribution.—Since relatively large amounts of radioactivity were present in liver 24 hours after administration of chlorozotocin-[ethyl-2-^{14}C] (0.75 mg/kg iv) to dogs (table 3), the subcellular distribution of the label in the liver was measured as described in the Methods section. Eighty percent of the ethyl-^{14}C was found in the soluble fraction (table 6), and almost 20% was sedimented with the nuclei. Although considerably less radioactivity was measured in liver 24 hours after administration of chlorozotocin-[glucose-1-^{14}C], the highest percentage of radioactivity was again found in the soluble fraction, but about 60% of the label was evenly distributed among the nuclei, mitochondria, and microsomes.

TABLE 7.—Distribution of radioactivity on chromatograms of rat bile and dog urine*

	Distribution of radioactivity on chromatogram (%)							
	R_f values							
	0.06 M_1	0.14 M_2	0.19 M_3	0.26	0.35	0.44 "impurity"	0.52 "chlorozotocin"	μg of chlorozotocin equivalents excreted/min
Glucose-^{14}C								
Rat bile								
5–10 mins	2.3	3.3	3.6	7.7	11.1	21.4	50.6	3.51
30–45 mins	4.3	7.7	4.8	11.1	14.7	40.8	16.6	0.94
Dog urine								
0–30 mins	0.7	1.1	1.8	4.0	16.8	23.8	51.8	102.00
3–6 hrs	1.1	2.4	3.3	23.3	50.8	15.9	3.3	4.85
Ethyl-^{14}C								
Rat bile								
5–10 mins	10.1	50.9	19.4	2.7	3.4	5.2	8.3	5.00
30–45 mins	3.2	70.4	24.3	0.8	0.4	0.5	0.5	7.74
Dog urine								
0–30 mins	1.8	2.1	7.7	4.3	23.0	24.6	36.4	77.70
3–6 hrs	7.7	5.9	28.2	5.5	29.5	19.3	3.9	4.31

*Bile from rats and urine from dogs collected after administration of [^{14}C]-chlorozotocin to the animals was chromatographed in butanol:acetic acid:water (3:1:1, vol/vol). Radioactivity was measured as described in the Methods section. Data shown are from a single representative animal in each group.

Chromatographic studies.—The nature of the radioactivity excreted in bile after the administration of [^{14}C]-chlorozotocin to rats and in urine after administration of the drug to dogs was investigated by TLC, as described in the Methods section. Authentic chlorozotocin, both the ethyl-^{14}C and glucose-^{14}C species, had an R_f of 0.52 in a butanol:acetic acid:water (3:1:1, vol/vol) solvent system. A small amount of an impurity (6%), which moved with an R_f of 0.44, was also observed in these preparations. Ten minutes after the administration of glucose-^{14}C-chlorozotocin to rats, one half of the radioactivity in bile was chromatographically indistinguishable from the unchanged drug (table 7). Approximately 20% of the radioactivity, however, had the same R_f (0.44) as the impurity in the original preparation. In subsequent bile samples the percentage of the radioactivity located at the R_f of the unchanged drug decreased and that observed at the R_f of the impurity became the predominant component although the actual amount of both components decreased. The presence of a third radioactive species (R_f of 0.35) also became apparent. The chromatographic profile of ^{14}C from a typical 45-minute sample is shown in table 7. The distribution of radioactivity on the chromatograms of urine from dogs 30 minutes following the iv administration of glucose-^{14}C-chlorozotocin was strikingly similar to that observed in rat bile collected at 10 minutes (table 7). For example, the early urine samples contained not only considerable amounts of radioactivity located at R_f 0.52, an R_f identical to that of authentic chlorozotocin, but also two other radioactive compounds with R_f values of 0.44 and 0.35. After several hours, however, the relative concentrations of the compound at R_f 0.52 and the compound with an R_f of 0.44 were decreased and those of the components at R_f values of 0.35 and 0.26 were markedly increased. When glucose-^{14}C-chlorozotocin was incubated in dog urine for 3 hours at 37°C, the chromatographic profile obtained from an aliquot of the incubation mixture (data not shown) was very similar to the profile obtained from the urine collected 30 minutes after administration of chlorozotocin to the dog. Also, when incubated in bile for 30 minutes at 37°C, much of the drug was converted to radioactive products which moved with R_f values of 0.44 and 0.35. Thus it appears likely that the redistribution of radioactivity on the chromatograms resulted from spontaneous decomposition of the drug rather than from the excretion of products. Following the administration of ethyl-^{14}C-chlorozotocin to rats, the radioactivity of three components other than the drug and impurity predominate in bile (tables 7 and 8). Since these compounds were not formed when the drug was incubated with bile it appears that they were products excreted in the bile. Moreover, these components were present in all of the bile samples, even those collected at 5 minutes after the administration of the drug when most of the radioactivity appeared to be in the form of chlorozotocin itself. The contribution of M_2 (R_f 0.14) to the radio-

TABLE 8.—R_f values of chlorozotocin-[ethyl-2-^{14}C] and its radioactive products*

Radioactive components in bile	R_f value in solvent system—		
	1	2	3
"Chlorozotocin"†	0.52	0.83	0.93
"Impurity"	0.44	0.75	0.84
M_1	0.06		0.03
M_2	0.14	0.08	0.07
M_3	0.19	0.15	0.12

*Samples of bile obtained from rats after administration of chlorozotocin-[ethyl-2-^{14}C] (5, 10, and 20 mg/kg iv) and [^{14}C]-chlorozotocin standards were chromatographed in 3 systems: 1 = silica gel G, butanol:acetic acid:water (3:1:1, vol/vol); 2 = silica gel G, acetone:water (85:15, vol/vol); 3 = cellulose, propanol:pyridine:acetic acid:water (15:10:3:12, vol/vol). Components were detected by autoradiography.

†R_f values of authentic chlorozotocin are identical to those of apparent chlorozotocin from bile in these solvent systems.

activity in bile increased rapidly and after 20 minutes accounted for 70% of the ^{14}C; only 25% appeared as M_3 (R_f 0.19) and < 1% as the unchanged drug. The profile of the biliary excretion of radioactivity remained essentially unchanged over the interval of 20–120 minutes after the administration of doses of 5–20 mg/kg of the ethyl-labeled drug. Only representative profiles for early (10 minutes) and later (45 minutes) samples are shown in table 7. Unlike those observed in rat bile, the temporal changes in the chromatographic profile of the radioactive components of R_f 0.52, 0.44, and 0.35 in dog urine after receiving the ethyl-^{14}C-labeled drug were almost identical to those seen after the glucose-^{14}C-labeled drug (table 7). Particularly noteworthy is the increase in the concentration of the components of low R_f in both rat bile and dog urine that follows the administration of ethyl-^{14}C-chlorozotocin.

DISCUSSION

Chlorozotocin is a new active antitumor agent which exhibits decreased bone marrow toxicity in mice (2,3). The importance of chlorozotocin in the treatment of patients with neoplastic disease will depend on it also exhibiting low myelosuppressive activity in man. In the present studies the levels of radioactivity were measured in the bone marrow of rats 6 hours after administration of either the glucose- or ethyl-labeled drug (10 mg/kg). The concentration of the ethyl-^{14}C species was 3.91 ± 0.35 ng/10^7 cells compared to 6.02 ng/10^7 cells measured in mice 1 hour after administration of a twofold higher dose of ethyl-^{14}C-chlorozotocin (9). However, it is noteworthy that when levels of ^{14}C in the bone marrow of rats were measured as μg of chlorozotocin equivalents/g of wet tissue, the concentration of ^{14}C from both the glucose- and ethyl-^{14}C species (4.41 ± 0.40 and 5.86 ± 2.11 μg/g, respectively) were twofold to threefold higher than the corresponding levels in plasma (1.33 ± 0.10 and 2.68 ± 0.64 μg/ml, respectively). A comparison of these values with the tissue levels found in rats with biliary cannulas (table 2) shows that concentrations of ^{14}C in bone marrow do not differ significantly from those found in liver, the tissue with the second highest concentration of ethyl-^{14}C. Although involving two groups of rats, this comparison appears to be valid since the plasma levels for both labels are comparable in the two experiments.

In spite of the apparent lack of myelotoxicity in mice, some toxicity might be anticipated in rats, especially at higher doses, since both labeled forms of chlorozotocin distribute to the bone marrow. Although levels of ^{14}C were not measured in the bone marrow of dogs, toxicology data[3] showed bone marrow hypoplasia and lymphoid atrophy at the highest doses administered (3 and 6 mg/kg). The most prominent toxicity in the dog was renal tubular damage with necrosis, which would correlate with the data presented in this paper showing a high renal excretion of ^{14}C and relatively high levels of ^{14}C in the kidney, 24 hours following administration of [^{14}C]-chlorozotocin to dogs (table 3).

The chloroethyl nitrosoureas are very reactive compounds which decompose rapidly, even in buffers at physiologic pH. Chlorozotocin differs structurally from CCNU by substitution of a molecule of glucose for its cyclohexyl moiety. The extensive chemical degradation of CCNU under physiologic conditions has been described by Reed et al (10), and some of the products have been identified in the urine of mice, dogs, and rats following administration of [^{14}C]-CCNU (11,12). Among the decomposition products of CCNU that have been identified are chloroethanol, acetaldehyde, vinyl chloride, ethylene, and cyclohexylamine. From its decomposition profile it was postulated that the degradation of CCNU involves the formation of cyclohexylisocyanate and 2-chloroethyldiazine hydroxide. Both of these species could be involved in the carcinostatic action of CCNU. For example, Reed et al (10) suggested that the 2-chloroethyldiazine hydroxide was the precursor of the alkylating species, possibly the 2-chloroethyl carbonium ion. The unstable cyclohexylisocyanate is very likely the species which reacts with proteins by carbamoylation of amino groups.

[3]Toxicology studies with chlorozotocin (NSC–178248) in beagle dogs and rhesus monkeys. Report to Laboratory of Toxicology, DTP, DCT, NCI, Feb 1976. US Department of Commerce National Technical Information Service, PB 254,569/AS.

We have found the nitroso group of chlorozotocin, like that of CCNU, to be very unstable at neutral or alkaline pH. For example, chlorozotocin can be assayed by procedures which use the nitroso group in the diazotization and coupling of amines. When chlorozotocin was incubated at 37°C in Tris buffer, pH 7.4, the $t_{\frac{1}{2}}$ of its nitroso group was about 30 minutes, and this rate of decomposition was not altered by incubation with liver homogenates. These findings are consistent with the view that the fate of chlorozotocin, like that of CCNU, involves the cleavage of the nitrosourea moiety. Indeed the disposition of the drug itself provides compelling evidence in support of this hypothesis. For example, the rate of excretion of radioactivity in urine 6 hours after the administration of the glucose-^{14}C-labeled drug to both rats and dogs is about twice that measured after the ethyl-^{14}C-labeled compound. Moreover, little of the glucose-^{14}C-labeled species is excreted in bile, but the excretion of the ethyl-^{14}C label is both rapid and extensive. Also noteworthy are the differences in both the levels and the subcellular distribution of radioactivity in liver. Twenty-four hours after the two labeled species were administered to dogs, the concentration of ethyl-^{14}C in liver was the highest of all the tissues examined and was about five times that of the glucose-^{14}C. The highest percentage of radioactivity from both labeled species was found in the soluble fraction. After the ethyl-^{14}C the remaining radioactivity was sedimented almost exclusively with the nuclei; that from the glucose-^{14}C was, however, distributed evenly among the nuclei, mitochondria, and microsomes.

A high percentage of the radioactivity found in the plasma after administration of both labeled species is associated with proteins. This association may be due in part to the alkylating activity of chlorozotocin and to a small degree to carbamoylation (13). The differences between the curves for the disappearance of total and unbound radioactivity from the plasma of dogs after administration of glucose-^{14}C-chlorozotocin are most striking. The unbound ^{14}C declined expectedly in a biphasic manner. The levels of total ^{14}C, however, after an initial rapid decline, actually increased and remained elevated for at least 4 hours, suggesting that after the uptake of chlorozotocin by the tissues a decomposition product may be released to the plasma and bound by protein. In fact, the small amount of glucose-^{14}C in plasma after 24 hours was almost entirely in the bound form. A similar rise in the levels of radioactivity in the plasma of monkeys was observed by DeVita et al (14) following iv administration of [^{14}C]-BCNU. A possible explanation, which could also be valid for chlorozotocin, was offered based on the solubility of BCNU. The suggestion was that the intact molecule entered the tissues and then a lipid insoluble fraction was released into the bloodstream to bind to protein.

It is possible to speculate concerning the nature of the excretion products of chlorozotocin from the chromatographic profiles of the radioactivity in urine and bile (table 7). The finding that appreciable amounts of radioactivity, which moved with an R_f of 0.26, was excreted in urine only after administration of the glucose-^{14}C-labeled drug suggests that this component has lost the ethyl group. Since the component with an R_f of 0.35 as well as the impurity which moved with an R_f of 0.44 were observed regardless of which labeled species was administered, it appears that these compounds retain both their glucose and ethyl moieties. Moreover, the observation that the three radioactive components with low R_f values (M_1, M_2, and M_3) were excreted in urine and bile only after administration of the ethyl-^{14}C-labeled drug is consistent with the view that they have lost the glucose moiety. Two of these low R_f components could be thiodiacetate and *S*-carboxymethyl-L-cysteine which have been identified as two metabolites of the chloroethyl moiety of CCNU in the rat (12).

The distribution and excretion of streptozotocin, the methyl nitrosourea analog of chlorozotocin, have been investigated in rats by Karunanayake et al (15–17). From the results of these studies it is evident that there are a number of similarities between the fate of the two drugs. For example, after the iv administration of methyl-^{14}C- or glucose-^{14}C-streptozotocin, radioactivity rapidly disappeared from plasma and accumulated in tissues. The majority of the injected radioactivity was associated with the liver and kidneys. The rates of urinary excretion of the two labeled species of streptozotocin for 6 hours after their administration were essentially the same as those observed in the present studies for glucose- and alkyl-labeled chlorozotocin. Moreover, it appears that streptozotocin like chlorozotocin undergoes extensive degradation prior to excretion.

The alkylating agents which are used in the chemotherapy of neoplastic diseases are characterized by their ability to form covalent linkages with compounds of biologic importance by reactions with a variety of nucleophilic substances including phosphate, hydroxyl, sulfhydryl, amino, carboxyl, and imidazole groups. In view of the instability of the substituted nitrosoureas at physiologic pH, it is indeed likely that chemical decomposition rather than metabolic transformation plays the more important role in determining the availability at sites of action of those reactive species which are respon-

sible for antineoplastic activity. Accordingly, with compounds such as chlorozotocin, information on chemical degradation as well as tissue distribution, intracellular localization, and binding to biologic material will be of importance in determining mechanism of action.

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Psychotropic Drugs as Potential Antitumor Agents: A Selective Screening Study [1]

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SUMMARY

Compounds with known psychotropic properties were tested for activity in murine ip L1210 leukemia and B16 melanoma in a protocol designed to obtain leads for new antitumor agents which might also possess central nervous system (CNS) antitumor properties. Barbiturates and hallucinogenic compounds were the only compound types deliberately excluded. Representatives from most of the other known CNS agent classes were included among the 297 psychotropic drugs evaluated. Sixteen of these agents were reproducibly active against the L1210 tumor system with T/C values of 125%–150%. Phenothiazines such as fluphenazine and butyrophenones such as triperidol were prominent among the confirmed active structural types. Dopamine, a β-phenethylamine neurotransmitter, was active. While reproducible B16 melanoma activity was not observed among the psychotropic drugs, most of the L1210 confirmed active agents were effective against the ip P388 tumor model and also were active in vitro against KB cells. Ic L1210 activity was not observed among the few compounds chosen for testing in that tumor system. The yield of ip L1210 confirmed actives from this group of psychotropic agents was 18 times that which would have been expected from the random screening of compounds.

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I. INTRODUCTION AND OBJECTIVES

The National Cancer Institute (NCI) has tested almost 300,000 compounds in animal tumor models over the past 20 years in its quest for new clinically useful anticancer drugs. Although random compound acquisition for this testing program no longer plays the major role it did several years ago, the biologic evaluation of new materials still has an important function in the development of new antitumor agent "leads." As the biology and biochemistry of the various cancer types become clearer, the ratio of rational to random compound input for testing should increase.

The study described here is an intermediate stage between purely random and completely rational selection of compounds for testing. The compounds selected all possess some type of central nervous system (CNS) activity. The general objective of this investigation was the discovery of a new antitumor agent lead. Psychotropic compounds were selected as the drug class to be evaluated in the hope that some of the compounds found to be active in standard ip tumor models might also possess CNS antitumor properties. There is a need for new CNS antitumor agents since brain tumors respond to few known drugs (1,2) and metastatic CNS leukemia often occurs in patients undergoing treatment for other types of leukemia (3–10).

There have been several reports on the interactive effects of CNS drugs and antitumor agents. Reserpine reduced the toxicity of nitrogen mustard *N*-oxide but chlorpromazine was without effect (11). Vinblastine but not vincristine potentiated the anesthetic effect of a barbiturate during tumor surgery (12). A number of psychotropic drugs were shown to be active against the murine Sarcoma 180 (S180) ascites tumor (13,14). Many of these compounds also potentiated the effect of cyclophosphamide in this tumor system. Cohen (15,16) has shown that a combination of caffeine and a psychotropic agent markedly enhanced the activity of BCNU against L1210 leukemia.

Bécause of these antitumor-related effects of psychotropic agents and the activity discovered in the general NCI screening program for several known CNS agents, a testing program was designed to evaluate the activity of a number of different types of psychotropic agents against several murine tumor models.

Also, it was anticipated that, in addition to the discovery of new antitumor-active compounds, we might evaluate the following hypothesis: a group of compounds which is known to have physiologic properties (in this case CNS activity) will yield a higher percentage of antitumor actives than randomly selected compounds.

II. EXPERIMENTAL METHODS

A. Selection of Agents for Testing

The requirement for a compilation of CNS-active agents from which compounds might be selected for testing was met by a monograph by Usdin and Efron (17). At the time this project was conceived (1971), the first edition of the Usdin and Efron work was available. The "psychotropic numbers" (P Nos.) used in the tables and text are the compound identification numbers from the first edition (17). Therefore, the structure of any compound corresponding to a P No. can be determined (*a*) from the name of the compound (appendix 1), or (*b*) by consulting reference 17. In 1972, a second expanded edition of Usdin and Efron was published (18) and the first edition went out of print. For this reason we have included the corresponding second edition P No. as "2nd Ed. No." in appendix 1.

Barbiturates were not included in reference 17 and were also excluded from our study. This appeared reasonable since > 700 of these compounds already had been tested in the NCI screening program and none of the barbiturates which had been tested in the L1210 leukemia and B16 melanoma systems were active. In addition, compounds described as hallucinogenic (17) were deliberately excluded from the study in order to protect the personnel conducting the animal tests. Otherwise, a good cross section of chemical classes were included (table 1).

B. Drug Acquisition

Approximately 500 of the compounds listed in reference 17 were requested for antitumor testing from 70 of the suppliers listed in that reference. Fifty-nine of the companies contacted responded (appendix 2). They were able to supply 297 of the requested compounds. These compounds are shown in appendix 1 which is arranged in P No. order. For convenience, a drug name versus P No. index (appendix 3) as well as an NSC No. versus P No. index (appendix 4) have been provided.

C. Antitumor Experiments

1. In vivo tumors

Two transplantable murine tumor models were chosen for the initial tests: (*a*) the ip implanted

TABLE 1.—Chemical classes tested

Compound type	P No.
Phenothiazines and related compounds	
Phenothiazines	2–84
Phenothiazine analogs and isosteres	87–140
Indoles	
Lysergic acid derivatives	145–154
Reserpine and derivatives	159–180
Harmine and derivatives	182
Other indole derivatives	199–235
Other heterocyclic compounds	
Chromone derivatives	245–248
Benzodiazepines	250–261
Other nitrogen heterocycles	263–467
Other nonnitrogen heterocycles	468–480
Aromatic compounds	
Phenylethylamine derivatives	482–541
Phenylacetic acid derivatives	551
Diphenylmethane derivatives	552–563
Benzoic acid derivatives	567–568
Benzodioxane derivatives	572–573
Other aromatic compounds	584–633
Aliphatic compounds	
Glycols	637
Carbamates	640–658
Carbinols	661
Amides and hydrazides	669–679
Amines and hydrazines	685
Other aliphatic compounds	688

L1210 leukemia, an ascites tumor which selects confirmed actives at the rate of about six per 2000 from among randomly acquired compounds (19) (this tumor, in retrospective studies, successfully predicted most of the early clinically active antitumor drugs [20]); and (*b*) the ip implanted B16 melanoma, a much more resistant solid tumor.

Both of these tumor models are in current use by the Developmental Therapeutics Program, NCI, and have been described in detail (21).

Compounds which proved to have confirmed L1210 activity were also tested in the P388 lymphocytic leukemia tumor system (21), a somewhat more sensitive tumor model which has recently supplanted L1210 as the NCI primary screen. Significant details of these three systems are as follows:

(*a*) *Ip L1210.*—BDF_1 or CDF_1 mice were inoculated ip with 10^5 L1210 cells and treated ip as indicated in figure 1. Activity is evaluated by determining the mean survival time of treated groups compared to control mice and expressed as a percentage (% T/C). A T/C value ≥ 125% is considered statistically significant activity (21).

(*b*) *B16.*—BDF_1 mice were implanted ip with 0.5 ml of a 1:10 g/ml tumor homogenate and the drug was administered ip each day for 9 days beginning 24 hours after implant. T/C activity values are based on median survival times (MST) with values ≥ 140% considered statistically significant.

(*c*) *P388.*—CDF_1 mice were inoculated ip with 10^6 cells from P388 ascitic fluid and treated ip on the best L1210 schedule. MSTs are calculated and T/C values ≥ 125% are considered statistically significant.

2. *In vivo protocols*

Figure 1 shows the testing protocol designed for this study. The initial experiment consisted of a five-dose assay in the L1210 system with drug administered ip on Days 1, 5, and 9 after tumor implant (q4d treatment schedule). Dose levels used were 400, 200, 100, 50, and 25 mg/kg/injection unless available rodent toxicity data indicated the use of lower doses. Four mice per test group (ie, per dose level) were employed in the initial q4d assay. The number of mice per test group was increased to six in all further L1210 assays and to ten in the B16 assays. An experiment (five-dose assay) was considered incomplete unless at least one dose was nontoxic (a dose which gave no early drug deaths) (21). If toxic, the experiment was repeated as necessary with adjusted dose levels. Doses for the L1210 and the B16 Days 1–9 treatment assays were based on data obtained from the original q4d experiment. Occasionally, when the supply of a drug became depleted before completion of protocol testing, the doses were reduced to the highest level allowing a complete five-dose assay, or the q4d schedule was substituted for the qd Days 1–9 B16 assay. Drugs active against L1210 on the q4d schedule were tested against B16 melanoma on both the q4d and qd Days 1–9 schedules. Drugs were administered ip in 0.9% saline or in Klucel (hydroxypropylcellulose) if insoluble.

When compounds gave L1210 T/C values ≥ 150%, they were tested against the ic implanted L1210 tumor system. Previous experience has shown that there is very little chance of obtaining significant ic activity when a T/C value of < 150% is obtained from ip testing in the same tumor model.

The basic L1210 and B16 melanoma tests were carried out at the Hazleton Laboratories, Vienna, Va, under NCI contract N01-CM-57007.

3. *In vitro tests*

Twenty-nine of the 30 compounds which gave at least one active L1210 test were evaluated in vitro for their ability to inhibit the growth of KB cells in culture (the supply of P-94 had been exhausted). This cell line originated in 1954 from a human epidermoid carcinoma of the mouth and has been used as a standard NCI cell culture screen for a

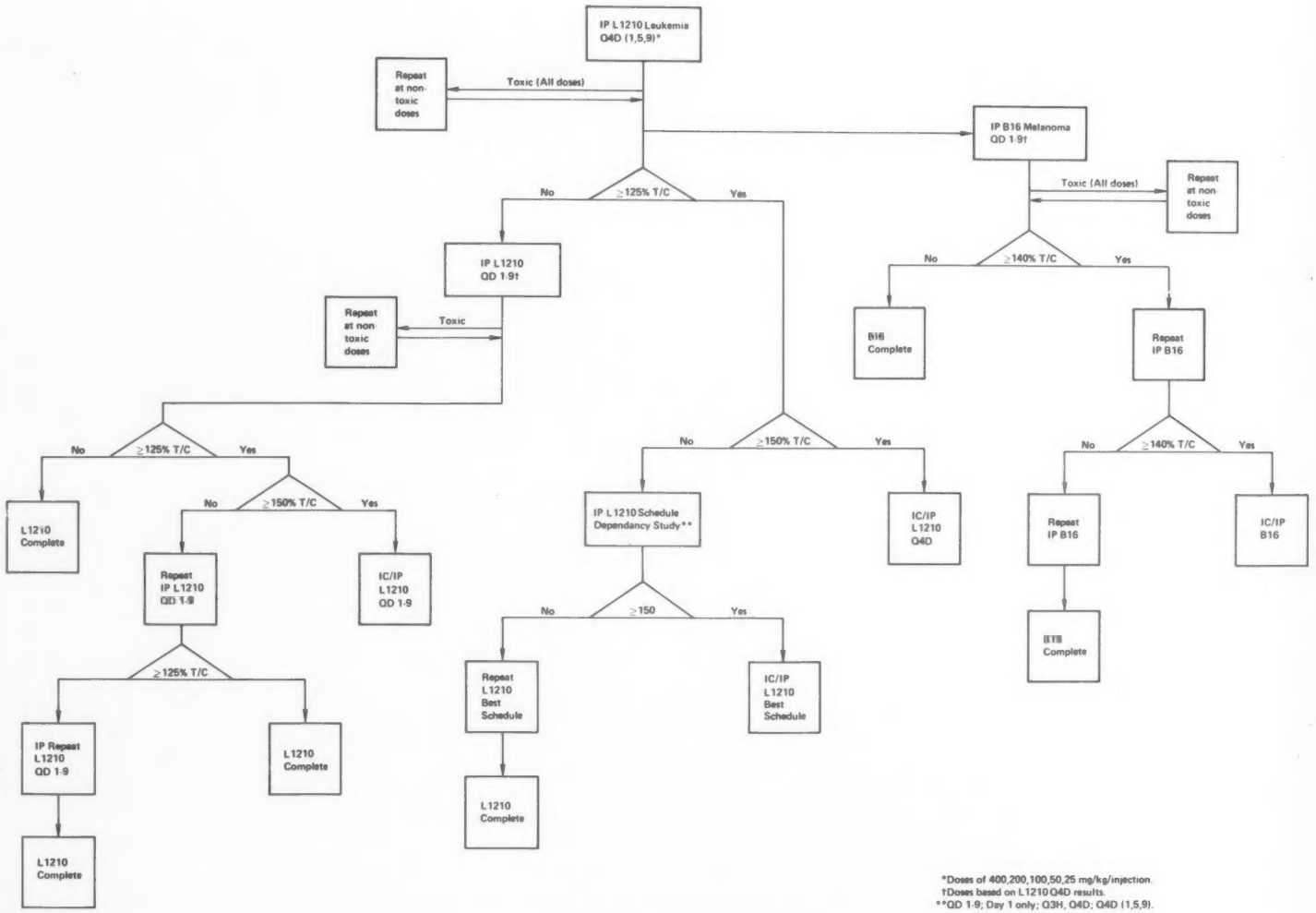


FIGURE 1.—Psychotropic drug antitumor testing protocol.

TABLE 2.—Compounds with confirmed activity against L1210 leukemia

Structural class	P No.	NSC No.	Structure in fig—	Active schedule†	No. of experiments	Optimum dose (mg/kg/injection)	%T/C*
Phenothiazine	2	169180	2	qd, 1–9	3	12.5	147
						25	133
						12.5	127
Phenothiazine	27	62323	2	1, 5, and 9	3	50	164
						25	130
						25	126
Phenothiazine	32	186057	2	1, 5, and 9	4	50	132
						50	128
Phenothiazine	80	17474	2	qd, 1–9	4	22	143
						50	130
Phenothiazine	81	17473	2	qd, 1–9	3	25	133
						25	132
Phenothiazine	120	169881	2	qd, 1–9	3	80	134
						75	132
						80	131
Phenothiazine isostere	91	169899	3	1	3	12	130
						60	125
Phenothiazine isostere	93	169185	3	1, 5, and 9	4	50	150
						25	135
Phenothiazine isostere	94	78714	3	1, 5, and 9	2	25	136
						50	133
Phenothiazine isostere	104	170952	3	1, 5, and 9	3	25	146
						40	146
						25	142
Phenothiazine isostere	121	169190	3	1, 5, and 9	3	50	132
						50	128
						25	128
Butyrophenone	320	186062	4	qd, 1–9	2	25	129
						25	126
Butyrophenone	459	169875	4	1, 5, and 9	5	50	145
						50	137
						25	134
						50	130
Phenethylamine	498	169105	4	qd, 1–9	3	400	152
						200	146
						200	133
Indole	235	107432	4	qd, 1–9	3	50	137
						25	125
Benzimidazole	375	169415	4	1, 5, and 9	3	100	141
						100	126

*%T/C = treated survival time/control survival time × 100%. T/C values for unreported tests (*see* No. of experiments) were < 125%.
†Treatment days.

TABLE 3.—Compounds with inconsistent activity against L1210 leukemia*

Structural class	P No.	NSC No.	Structure in fig—	Active schedule	No. of experiments	Optimum dose (mg/kg/injection)	%T/C
Phenothiazine	13	17479	2	1, 5, and 9	2	50	148
				qd, 1–9	3	6.25	127
Phenothiazine	60	169450	2	1, 5, and 9	2	100	130
				qd, 1–9	3	50	129
Phenethylamine	417	33379	4	1	2	75	147
				qd, 1–9	3	4.1	132
				1, 5, and 9	1	12.5	125
Phenethylamine	486	27104	4	1	2	56	138
				1, 5, and 9	3	9.4	136

*See table 2 for definitions.

number of years (21). Table 5 shows these KB data. The biologic response is the concentration required to inhibit cell growth to 50% of control growth by 3 days after drug addition.

III. RESULTS

A. Introduction

The compounds which were ip L1210 active in more than a single test are shown in tables 2 and 3. P388 in vivo and KB in vitro results for these compounds are given in tables 4 and 5 respectively. The structures of the actives, organized by compound class, are shown in figures 2–4.

Table 2 indicates compounds with confirmed L1210 activity on a given treatment schedule. Table 3 shows compounds which gave two or more active tests, but not on the same treatment schedule. Table 4 gives the P388 results for those compounds which had confirmed activity against the L1210 tumor. The L1210 active treatment schedules were used in the P388 tests.

None of the compounds in this study gave reproducible, statistically significant (T/C $\geq$ 140%) B16 melanoma activity.

According to the protocol under which this study was conducted (fig 1), dopamine (P-498) and fluphenazine (P-27) met the criterion for ic L1210 testing. With the exception of one ic experiment with P-27 (T/C 143%), both compounds were inactive in this system. This was not an unexpected result since (*a*) the ip L1210 activity was too marginal to expect ic L1210 activity, and (*b*) it is known that dopamine does not readily cross the blood-brain barrier (22) and therefore would not be expected to have ic activity.

B. Phenothiazines

1. Introduction

The majority of the compounds discovered to possess L1210 activity in this study were phenothiazines and phenothiazine isosteres. Phenothiazine derivatives are well known as CNS agents (23,24). Thousands of these compounds have been prepared (23,25) and evaluated, primarily as major tranquilizers and antipsychotic agents.

Several reports also allude to the possibility that these compounds have antitumor properties. Chlorpromazine (P-13) is, by far, the best studied of these agents. Antitumor effects have been claimed for this compound in cancer patients undergoing treatment for schizophrenia (26,27). P-13 reduced the tumor weight in a mouse mastocytoma (28), was effective against Sarcoma 37 (29), reduced the incidence of dimethylbenz[a]anthracene (DMBA)-induced hamster cheek pouch tumors (30), and protected against carcinogen-induced adrenal necrosis (31). This compound was also active against sc B16 melanoma (32), weakly active against ascites S180 (13), and reduced the antitumor effects of stress on DMBA-induced tumors (33). Synergistic effects of chlorpromazine with known antitumor agents have also been noted. When given with caffeine and BCNU, P-13 increased cure rates substantially in the L1210 system (15,16). It was reported to enhance the activity of nitrogen mustard (34), but did not reduce the toxicity of nitrogen mustard *N*-oxide (16). Although other phenothiazines increased the effectiveness of cyclophosphamide, P-13 had little effect (13,14,34).

In addition to chlorpromazine (P-13), several other members of this family, including thiethylperazine (P-74) (14), fluphenazine (P-27) (35), chlorprothixene

TABLE 4.—P388 leukemia activity*

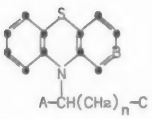
Structural class	P No.	NSC No.	Structure in fig—	Active schedule	No. of experiments	Optimum dose (mg/kg/injection)	%T/C
Phenothiazine	2	169180	2	qd, 1–9	2	—	Not active
Phenothiazine	27	62323	2	1, 5, and 9	3	50	166
						25	145
				qd, 1–9	3	1.9	136
						1.25	126
Phenothiazine	32	186057	2	1, 5, and 9	1	—	Not active
Phenothiazine	80	17474	2	1, 5, and 9	5	100	163
						100	150
						37.5	134
						37.5	130
Phenothiazine	81	17473	2	qd, 1–9	4	12.5	145
						25	128
						16.5	127
				1, 5, and 9	2	50	143
						50	140
Phenothiazine	120	169881	2	qd, 1–9	2	100	137
						100	133
Phenothiazine isostere	91	169899	3	1	1	—	Not active
Phenothiazine isostere	93	169185	3	1, 5, and 9	1	12.5	134
				qd, 1–9	3	25	135
						25	126
Phenothiazine isostere	104	170952	3	1, 5, and 9	2	40	145
						40	135
Phenothiazine isostere	121	169190	3	1, 5, and 9	3	50	147
						50	145
Butyrophenone	320	186062	4	qd, 1–9	1	25	124
Butyrophenone	459	169875	4	1, 5, and 9	2	—	Not active
Phenethylamine	498	169105	4	qd, 1–9	3	600	168
						600	153
						300	139
Indole	235	107432	4	qd, 1–9	1	—	Not active
Benzimidazole	375	169415	4	1, 5, and 9	2	200	135

*P388 tests conducted only on confirmed actives in the L1210 system (table 2). P94 not tested due to exhausted supply. *See* table 2 for definitions.

(P-91) (13), and prochlorperazine (13,36), are reported to have antitumor effects. Phenothiazine, the parent ring system, was protective against DMBA-induced tumors (31,37). Phenothiazines containing alkylating groups have been used with varying degrees of success against the murine S180, Walker 256 carcinosarcoma, P388, and L1210 tumor models (38–41).

2. *L1210 lymphoid leukemia activity*

Six phenothiazines and five phenothiazine isosteres were confirmed active (ie, reproducibly active on a given schedule) in the L1210 system (table 2). Fluphenazine (P-27), acetophenazine (P-2), trifluoperazine (P-80), clopenthixol (P-93), and flupentixol (P-104) had T/C values > 140% in at least one test. Eight of the 11 confirmed actives contain the piperazine ring in the N-10 side chain. This side chain is very common among the phenothiazines with better CNS properties (tranquilizing, antipsychotic, antiemetic) than autonomic properties (antihistaminic). Most of the antitumor active compounds contain a lipophilic, electronegative group in the 2-position of the ring. Substitution of trifluoromethyl or chloro in the 2-position enhances CNS activity relative to peripheral autonomic action (42). Both the pipera-



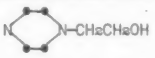
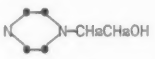
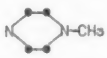
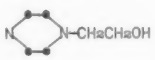
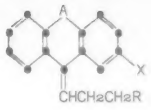
P Number	Name	Biological Activity in Table	A	B	C	n	Salt
2	Acetophenazine	2,4	H	C-$COCH_3$		2	2 maleate
13	Chlorpromazine	3	H	C-Cl	$N(CH_3)_2$	2	HCl
27	Fluphenazine	2,4	H	C-CF_3		2	2 HCl
32	KS-33	2	H	C-OCH_3		1	
60	Propiomazine	3	CH_3	C-COC_2H_5	$N(CH_3)_2$	1	HCl
80	Trifluoperazine	2,4	H	C-CF_3		2	2 HCl
81	Triflupromazine	2,4	H	C-CF_3	$N(CH_3)_2$	2	HCl
120	Oxyperpendyl	2,4	H	N		2	2 HCl

FIGURE 2.—Active phenothiazine derivatives.



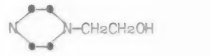
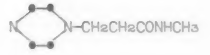
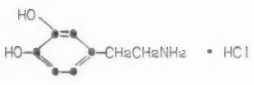
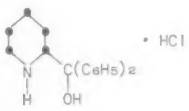
P Number	Name	Biological Activity in Table	A	X	R	Salt
91	Chlorprothixene	2,4	S	Cl	$N(CH_3)_2$	HCl
93	Clopenthixol	2,4	S	Cl		2 HCl
94	Clothixamide	2	S	Cl		2 maleate
104	Flupentixol	2,4	S	CF_3		2 HCl
121	P-5227	2,4	CH_2O	Cl		2 HCl

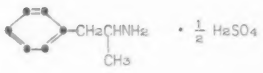
FIGURE 3.—Active phenothiazine isosteres.



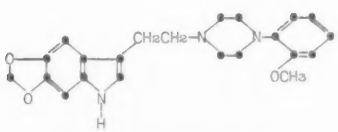
P498 (Dopamine, T2,4*)



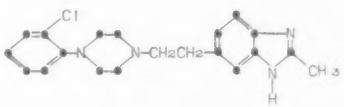
P417 (Pipradol, T3)



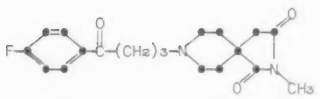
P486 (D-Amphetamine, T3)



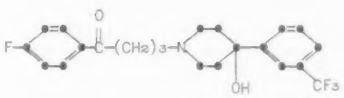
P235 (Solypertine, T2,4)



P375 (M&B 7150, T2,4)



P320 (FR-33, T2,4)



P459 (Triperidol, T2,4)

*Tables with antitumor data

FIGURE 4.—Other active compounds.

TABLE 5.—KB cell culture response

P No.	Compound type	ED50 (μg/ml)*
5	Phenothiazine	3
32	Phenothiazine	3
36	Phenothiazine	3
93	Phenothiazine isostere	3
104	Phenothiazine isostere	4
74	Phenothiazine	4
80	Phenothiazine	4
57	Phenothiazine	4
27	Phenothiazine	4
13	Phenothiazine	5
81	Phenothiazine	6
120	Phenothiazine	11
121	Phenothiazine isostere	11
60	Phenothiazine	11
2	Phenothiazine	14
235	Miscellaneous	22
91	Phenothiazine isostere	23
263	Butyrophenone	25
317	Butyrophenone	25
498	Phenethylamine	25
459	Butyrophenone	27
323	Butyrophenone	27
445	Butyrophenone	30
417	Phenethylamine	36
451	Miscellaneous	> 100
486	Phenethylamine	> 100
375	Miscellaneous	> 100
436	Miscellaneous	> 100
320	Butyrophenone	> 100

*Dose which inhibits cell growth to 50% of that of control growth by 3 days after drug addition.

zine ring and the 2-substituents increase the lipophilic nature of these molecules. This property, as well as other structural parameters, has been correlated with phenothiazine CNS activity (23,42,43).

Chlorpromazine (P-13) showed inconsistent antitumor activity (nonreproducible activity on two different treatment schedules, table 3). Its 2-trifluoromethyl analog, triflupromazine (P-81), was reproducibly active (table 2), while promazine, with no 2-substituent, was not reproducibly active. Analogs of P-13 containing the 2-OCH_3 (P-41), 2-SCH_3 (P-75), and 2-COC_2H_5 (P-61) were inactive. Addition of a 4-chloro substituent (P-19) or a sulfoxide oxygen (P-14) to P-13 produced inactive materials.

Fluphenazine (P-27) had been described earlier as active against the ascites S180 (14), R3230AC mammary carcinoma (35), and L1210 leukemia (44) systems. In the present study it was active in three of three L1210 tests giving a maximum of 164% T/C. Modification of this molecule to produce a decanoate ester (P-26) or a homophenazine (seven-member side chain ring, P-29), or replacement of the CF_3 with a chloro group to give perphenazine (P-49) abolished the L1210 activity of P-27. Oxyperpendyl (P-120), which possesses the fluphenazine structure with a heterocyclic nitrogen atom replacing the P-27 carbon and trifluoromethyl group in the ring 2-position (fig 2), was reproducibly active. However, alteration of the oxyperpendyl side chain to give P-134 abolished the activity.

Trifluoperazine (P-80) was active with a T/C value of 143%. Replacement of the methylpiperazine ring with the dimethylamino group gave triflupromazine (P-81) which had approximately the same level of activity as P-80. This was in contrast to the result obtained by making the same type of replacement in the thiethylperazine series (P-74 versus P-75). The addition of a methyl group to the propylamino side chain of P-80 resulted in an inactive compound (P-70).

The *N*-methylpiperidyl derivative, P-32, gave T/C test values of 132% and 128%. This compound contains a 2-methoxy substituent (fig 2). If the 2-substituent is changed to isopropoxy (P-33), bromo (P-31), chloro (P-55), or methylmercapto (P-78), inactivity results. The analog with the 2-methylsulfinyl group (P-36) was active in a single test (T/C 130%).

Thiethylpyrazine (P-74) had been found to possess excellent activity against ascites S180 in an earlier study (18). This compound was active (T/C 146%) in only one of three L1210 tests.

3. *P388 lymphocytic leukemia activity*

The six phenothiazines with confirmed L1210 activity (table 2) were tested against the P388 tumor model (table 4). Although acetophenazine (P-2) and KS-33 (P-32) were not active in limited testing, fluphenazine (P-27), trifluoperazine (P-80), and triflupromazine (P-81) were quite active giving T/C values > 160% in some tests. Oxyperpendyl (P-120) was reproducibly active also.

4. *KB cell culture activity*

All the phenothiazines showing L1210 activity in at least one in vivo experiment were tested in the KB system (table 5). This compound family was uniformly active with ED50 values in the range of 3–14 μg/ml. Seven of these compounds had response values of ≤ 4 μg/ml. This activity is consistent with the known cell membrane properties of the phenothiazines (45).

C. Phenothiazine Isosteres

1. *L1210 lymphoid leukemia activity*

Five phenothiazine isosteres in which the heterocyclic nitrogen atom was replaced by a double-bonded carbon (fig 3) were found to have L1210 activity. All were confirmed active materials (table 2).

Chlorprothixene (P-91) is active and an analog of chlorpromazine (P-13). The similar analog of fluphenazine, flupentixol (P-104), was also active with a T/C value of 146% (table 2). Substitution of a 2-chloro for a 2-trifluoromethyl group in P-104 gave clopenthixol (P-93) with undiminished activity. Replacement of the sulfur atom in P-93 with a methyleneoxy group (P-121) gave a compound which retained activity. However, replacing the hydroxyethylpiperazine group in P-121 with the dimethylamino group to give doxepin (P-101) resulted in inactivity. Imipramine (P-109) and its chlorpromazine analog chlorimipramine (P-90) were inactive. Imipramine had earlier been shown to be active in the S180 system (13,14). If the double bond in clopenthixol (P-93) is reduced to give xanthiol (P-104), activity is lost.

Among the 36 phenothiazine isosteres tested (table 1), ten had the heterocyclic sulfur atom replaced. None of these compounds were active. Fourteen had both the ring nitrogen and sulfur atoms replaced. Only one of these compounds (P-121) was active. However, when the nitrogen atom was replaced, four of the eight analogs in this category were active (fig 3) and two of the four inactive compounds had undergone additional structural modifications (P-125 and P-140).

2. P388 lymphocytic leukemia activity

Chlorprothixene (P-91) was inactive in the only test of this compound against the P388 tumor model. Clothixamide (P-94) was not tested because the supply of compound had been exhausted during the L1210 studies. The other three L1210 active compounds (P-93, P-104, and P-121) were active in the P388 system (table 4).

3. KB cell culture activity

Phenothiazine isosteres showed a level of KB activity comparable to that of the phenothiazines. The four compounds evaluated possessed ED50 values of 3, 4, 11, and 23 μg/ml (table 5).

D. Butyrophenones

1. Introduction

The butyrophenones are part of the class called "other nitrogen heterocycles" in table 1. These compounds are well known for their neuroleptic properties and have been extensively reviewed and compared with the phenothiazines which produce similar CNS effects (23,43).

Haloperidol (P-323), the most completely studied of these compounds, and trifluperidol (triperidol, P-459) were previously tested against the ascites S180 tumor model. While the former compound was active, the latter was not (14). P-323 was found to increase serum prolactin levels in studies with a DMBA-induced mammary tumor (46).

2. L1210 lymphoid leukemia activity

Fifteen butyrophenones were tested in this study. Six of these compounds (P-263, P-317, P-320, P-323, P-445, and P-459) showed activity in at least one experiment, with two, triperidol (P-459) and FR-33 (P-320), producing reproducible activity (table 2).

Triperidol (P-459) was the most active of the compounds of this type with four of five experiments giving activity (T/C 145%, 137%, 134%, and 130%). Replacement of the trifluoromethyl function in P-459 with a methyl group (P-384) abolished activity. The two spiro derivatives tested (P-320 and P-445) were active. Haloperidol (P-323), a relative of the active compound triperidol (P-459), gave an antitumor effect in one of three tests (T/C 134%). Except for P-320, the direct attachment of a carbonyl group in the 4-position of the piperidine ring resulted in inactive derivatives (P-322, P-377, P-403, and P-425). The piperazine (P-287 and P-318) and benzimidazolinone (P-281 and P-305) analogs were not active.

3. P388 lymphocytic leukemia activity

Neither of the two L1210 confirmed active compounds (P-320 and P-459) had activity against the P388 system in limited testing (table 4).

4. KB cell culture activity

The four butyrophenones tested were less cytotoxic than the phenothiazine analogs. P-320, which had confirmed L1210 activity, was inactive (ED50 > 100 μg/ml) in this test. The other three analogs (P-323, P-445, and P-459) fell into the activity range of 27–30 μg/ml.

E. β-Phenethylamines

1. Introduction

β-Phenethylamines play a well-established role as neurotransmitter substances. There are also some reports that these compounds or their precursors have antitumor activity or affect the activity of other agents. Dopa, dopamine (P-498), noradrenalin (norepinephrine), and adrenalin (epinephrine) all were shown to have an antitumor effect on the murine S180 tumor (47). The neurotoxic compound, 6-hydroxydopamine, slowed the growth of C1300 neuroblastoma and the A10 breast adenocarcinoma but was ineffective against B16 melanoma (48). Norepinephrine (P-520) was found to increase the

blood supply to a rat fibrosarcoma and when combined with methotrexate caused a reduction in tumor size (49). Dopamine (P-498) stimulated adenylate cyclase activity in malignant neuroblastoma cells (50). Serotonin doubled the effectiveness of nitrogen mustard and cyclophosphamide against the Ehrlich ascites tumor in vivo but did not potentiate 5-fluorouracil or thioTEPA (34). Dopa was found to abolish the protective effect of reserpine on nitrogen mustard *N*-oxide toxicity (11).

2. L1210 lymphoid leukemia activity

Twenty-eight phenethylamines (table 1) were evaluated. Dopamine (P-498) was confirmed active (table 2), while D-amphetamine (P-486) and piperadol (P-417) showed inconsistent activity (table 3). The structures of these compounds are shown in figure 4.

Dopamine (P-498) was active in all three of the L1210 experiments performed giving T/C values of 152%, 146%, and 133%. A multiple-dose (qd Days 1–9) treatment schedule was required. The doses used did not appear to be toxic to the test animals. Although not included in this psychotropic study, dopa, epinephrine, tyramine, and β-phenethylamine had been shown to be L1210 inactive in previous NCI testing. Methyldopa (P-515) and norepinephrine (P-520) were not active.

D-Amphetamine (P-486) produced one active test on the Day 1 treatment schedule and one on the Days 1, 5, and 9 schedule (table 3). Racemic amphetamine (P-485) was inactive as was methamphetamine (P-512), *p*-hydroxyamphetamine (P-502), two hydrazine analogs (P-508 and P-528), and a cyclopropyl analog (P-541).

Pipradol (P-417) was also inconsistently active on three different schedules, giving T/C values of 147%, 132%, and 125%. α-Hydroxy-β-phenethylamines which did not show activity were L-ephedrine (P-499), *m*-hydroxynorephedrine (P-504), phenylephrine (P-530), and synephrine (P-538). The structure-activity relationships among the β-phenethylamines and their analogs are currently under investigation.

3. P388 lymphocytic leukemia activity

Dopamine (P-498) was active in three of three experiments giving T/C values of 168%, 153%, and 139%.

4. KB cell culture activity

Dopamine (P-498) and pipradol (P-417) had ED50 values of 25 and 36 μg/ml respectively. D-Amphetamine, which possessed inconsistent L1210 activity (table 3), was inactive in this in vitro system.

F. Other Chemical Classes

1. L1210 lymphoid leukemia activity

An indole (solypertine, P-235) and a benzimidazole (P-375) were found to have confirmed L1210 activity (table 2). Both of these compounds contain the same type *o*-substituted-*N*-phenylpiperazine side chains (fig 4).

P-436, which is similar in structure to the side chain of the reproducibly active butyrophenone (P-320), had nonreproducible L1210 activity (activity in a single test) as did tetramethylsuccinimide (P-451).

A number of other well-known CNS agents were inactive using these protocols. Yohimbine (P-181), thalidomide (P-452), phenytoin (P-412), chlordiazepoxide (P-250), diazepam (P-251), amitriptylene (P-87), imipramine (P-109), iproniazid (P-328), and isoniazid (P-330) were all L1210 inactive. Oxypertine (P-226), a known neuroleptic drug with some physiologic properties characteristic of phenothiazines (45), was inactive, although an analog, P-235, possessed confirmed activity.

Serotonin, which was not included in this study, was reported to inhibit Sarcoma 45 (51) and the solid, but not the ascites, Yoshida sarcoma (52). It also affected the M1 sarcoma as well as the RS1 hepatoma (53). Serotonin was shown to have no L1210 activity in earlier NCI tests.

Previous reports indicated that reserpine (P-173) reduced the toxicity of nitrogen mustard *N*-oxide (11), reduced the growth rate of Sarcoma 37 (29), and showed activity in the L1210 system at 24°C but not 25°C (54). However, reserpine was inactive using our L1210 protocol.

2. P388 lymphocytic leukemia activity

Of the two compounds in this group with reproducible L1210 activity, only P-375 gave an active P388 test (T/C 135%).

3. KB cell culture activity

As a group, these compounds had the poorest cytotoxic effects of any in the study. Only solypertine (P-235) with an ED50 of 22 μg/ml showed any activity. The other three compounds evaluated had values of > 100 μg/ml.

IV. DISCUSSION

The physiologic effects of the phenothiazines, and especially chlorpromazine (P-13), have been studied in detail (23,45). Although the mechanism and sites of action for these drugs are largely still unknown, they produce a very large spectrum of biologic effects in vivo and in vitro. Chlorpromazine is

known to form free radicals (42,55), charge-transfer complexes with naturally occurring substances (42,56), and to complex with many CNS-active materials (23). Phenothiazines are good surface-active and hemolytic agents (45,57), and they have been tested with known antitumor agents (34) and other compounds (23,42) to increase cell membrane permeability.

The butyrophenones, like the phenothiazines, are neuroleptic agents (23). Structural similarities of these compounds with the phenothiazines have been noted, suggesting a common target site (43). No previous reports of antitumor activity among the butyrophenones were found.

Dopamine (P-498) and D-amphetamine (P-486), compounds found here to be L1210 active, were previously shown to have interdependent relationships with some of the other active compounds in this study. Psychotropic drugs in general (58,59) and phenothiazines in particular (23,43,45,60,61) affect the concentrations of β-phenethylamines and particularly dopamine. An excellent discussion of the interrelationships among psychotropic drugs, neurotransmitter amines, and schizophrenia has appeared recently (62). Whether any of these interdependencies have a bearing on the antitumor activity observed here will have to wait until additional information is available.

The temperature-lowering property (hypothermia) of CNS agents must be considered as a potential mechanism of antitumor action. This is a property of the phenothiazines (55,63) which has been reviewed for chlorpromazine (P-13) (23). Fondy et al (54) showed, that under certain experimental conditions, hypothermic effects could explain all of the antitumor activity produced by reserpine (P-173) in their L1210 experiments. Antitumor activity in those experiments was dependent upon the ambient temperature to which the mice were exposed. When mice bearing the L1210 tumor were treated with reserpine (P-173) in this study, no antitumor activity was observed. In addition, during the period 1962–1976, the NCI tested reserpine in 23 separate experiments using five different treatment schedules with multiple doses. L1210 activity was never obtained with reserpine. Activity was also not observed with this compound in the P388 leukemia and B16 melanoma systems. While hypothermia can not be discounted as a contributor to the antitumor properties of the psychotropic compounds which were found to be active, the inactivity of reserpine (P-173) and the marginal antitumor activity of chlorpromazine (P-13) in this study must minimize the importance of this explanation based on the information presently available. Until biochemical and pharmacologic studies are undertaken, the mechanisms of psychotropic drug antitumor activity (membrane effects, enzyme or naturally occurring compound interaction, hypothermia, etc) can only be speculated upon.

The hypothesis that a group of compounds with known physiologic properties (in this case CNS properties) might produce a higher percentage of antitumor actives than randomly selected compounds appears to be substantiated. The expected yield of L1210 confirmed actives from the random screening of compounds is approximately six per 2000 compounds tested or 0.3% (19). From the present study, the following points can be made:

1. A yield of 16 confirmed L1210 actives from the 297 compounds tested is 18 times higher than would be expected from random screening;
2. Since the phenothiazines influence the statistics to a great extent, it is of interest to examine the results of this study without these compounds included. Removal of the 78 phenothiazines and their 11 confirmed actives gives five confirmed actives (2.3%) from the 219 remaining compounds. This is still eight times the yield expected from random screening.

Twenty-nine of the 30 compounds with L1210 activity (tables 2 and 3) were evaluated in vitro in the KB cell culture system (table 5). All the phenothiazine-type antipsychotics were cytotoxic. With the exception of two compounds (P-320 and P-375), all the drugs reproducibly active against L1210 leukemia (table 2) had ED50 values ≤ 27 μg/ml.

Based on the activity found in this study, several compounds (P-27, P-80, P-93, P-104, P-320, P-459, and P-498) currently are being evaluated further in a panel of new tumor models (64).

V. CONCLUSIONS

Relative to the objectives of this study as stated in section I, the following conclusions can be drawn:

A. Sixteen psychotropic agents were found to possess moderate, but reproducible, activity in the ip L1210 leukemia system from among the 297 compounds tested. This yield of actives is 18 times that which would be expected from the screening of randomly acquired compounds.

1. The psychotropic drugs classed as neuroleptic agents (also known as antipsychotic agents, antischizophrenic agents, and major tranquilizers) accounted for 13 of 16 confirmed actives.
   a. Phenothiazines and their bioisosteres provided 11 of the L1210 confirmed active neuroleptics. These compounds are acetophenazine (P-2), fluphenazine (P-27), KS-33 (P-32), trifluoperazine (P-80), triflupromazine (P-81), chlorprothixene (P-91), clopenthixol (P-93),

clothixamide (P-94), flupenthixol (P-104), oxyperpendyl (P-120), and P-121.

b. Two butyrophenones, FR-33 (P-320) and triperidol (P-459), accounted for the other nonphenothiazine L1210 active neuroleptics.

2. Three additional compounds possessed confirmed L1210 activity.

a. A β-phenethylamine neurotransmitter, dopamine (P-498), possessed good ip L1210 activity (T/C 152% and 146%).

b. An indole, solypertine (P-235), and a benzimidazole (P-375), both containing an arylpiperazinylethyl side chain, were reproducibly active. Solypertine may have neuroleptic properties since an analog, oxypertine (P-226), is classed as this type of agent (45).

B. Four additional compounds (table 3) were found to exhibit some less reproducible level of ip L1210 activity.

C. No compounds with CNS antitumor properties were discovered. The level of L1210 ip activity (T/C 125%–150%) among the reproducibly active compounds was too low to expect corresponding ic L1210 antitumor activity. Ip B16 melanoma activity was not observed.

D. Ten of the compounds reproducibly active against the ip L1210 system were also active in the ip P388 leukemia tumor model.

E. Twenty-nine compounds were tested in the KB cell culture system. The phenothiazines and their isosteres possessed significant cytotoxic properties.

F. It appears that, as a class, psychotropic drugs possess a much higher percentage of L1210 actives than do randomly obtained compounds. This suggests that selective screening of other classes of compounds with specific biologic activities might be fruitfully undertaken to discover new antitumor agent leads.

G. The utility of the active agents described in this study will not be known until basic studies are done to determine the mechanisms of antitumor activity. The moderate activity level exhibited thus far by the confirmed actives would suggest limited possibilities for these materials as single agents in cancer therapy. However, the known ability of these materials to alter membranes and form complexes in biologically important systems might suggest a role for some of these compounds in combination treatments for cancer.

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APPENDIX 1. PSYCHOTROPIC DRUGS EVALUATED

P No.	2nd. Ed. No.	NSC	NAME
2	4	169180	ACETOPHENAZINE MALEATE
3	6	169468	AMINOPROMAZIN(E) FUMARATE
5	9	71755	CARPHENAZINE
12	19	169470	CHLORPROETHAZINE
13	20	17479	CHLORPROMAZINE
14	22	170990	CHLORPROMAZINE SULFOXIDE
19	35	172128	DICHLORPROMAZIN(E)
21	38	169183	DIMETHOXANATE
22	43	169095	DIXYRAZINE
23	96	169467	ETHOPROPAZIN(E)
24	44	169471	ETHYLISOBUTRAZINE
26	46	169510	FLUPHENAZINE DECANOATE
27	47	62323	FLUPHENAZINE DIHYDROCHLORIDE
29	50	169880	HOMOPHENAZINE
31	59	186058	KS-24
32	60	186057	KS-33
33	61	186056	KS-75
36	65	186066	MESORIDAZINE
37	66	169091	METHDILAZIN(E)
41	72	169469	METHOXYPROMAZINE MALEATE
43	76	169877	NDR A-3680
49	86	169181	PERPHENAZIN(E)
50	87	169182	PERPHENAZINE SULFOXIDE
51	90	54546	PHENETHIAZINE
55	94	186055	PIPERIDOCHLORPROMAZINE
57	98	17468	PROMAZIN(E)
58	99	30321	PROMETHAZIN(E)
60	101	169450	PROPIOMAZIN(E)
61	102	169500	PROPIOPROMAZINE
64	107	169466	RP 3300
66	110	169473	RP-7360
67	111	169475	RP-9153
68	112	169476	RP-9260
70	116	50440	SKF 5657
74	121	186068	THIETHYLPERAZINE MALEATE
75	122	186063	THIOMETHYLPROMAZINE
78	125	186060	THIORIDAZIN(E) HYDROCHLORIDE
80	130	17474	TRIFLUOPERAZIN(E)
81	132	17473	TRIFLUPROMAZIN(E)
83	134	170991	TRIEMPRAZIN(E) TARTRATE
84	136	169096	UCB 2493
87A	156	169910	AMITRIPTYLINE HYDROCHLORIDE
87B	156	176555	AMITRIPTYLINE N-OXIDE HYDROCHLORIDE
88	162	169184	BUTRIPTYLINE
89	163	169864	CARBAMAZEPIN(E)
90	165	169865	CHLORIMIPRAMINE
91	168	169899	CHLORPROTHIXENE
93	173	169185	CLOPENTHIXOL
94	175	78714	CLOTHIXAMIDE MALEATE
95	179	169911	CYPROHEPTADINE
98	184	169112	DIBENZEPIN HYDROCHLORIDE

APPENDIX 1 (continued)

P No.	2nd. Ed. No.	NSC	NAME
99	185	169431	DEPTROPINE CITRATE
100	186	100297	DIMETHACRIN(E)
101	187	108160	DOXEPIN
104	191	170952	FLUPENTIXOL
108	205	186059	ID 22
109	206	169866	IMIPRAMINE
110	207	169424	IMIPRAMINE N-OXIDE
111	780	169186	ISOTHIPENDYL
115	217	169092	MJ 1997-1
118	223	169453	NORTRIPTYLINE HYDROCHLORIDE
119	227	169867	OPIPRAMOL
120	878	169881	OXYPERPENDYL
121	229	169190	P-5227
123	233	173379	PROHEPTATRIENE
124	234	172129	PROPAZEPINE
125	235	172130	PROTHIADENE
127	236	169912	PROTRIPTYLINE
128	237	169900	RO 4-6011
129	238	169474	RP 8307
131	240	170992	SKF 10810
132	241	170994	SKF 10,812
133	242	111699	SQ 10,638
134	878	106961	THIOPHENYLPYRIDYLAMIN(E)
135	243	108165	THIOTHIXENE
137	245	169472	TRIMEPRAMINE
138	246	169097	UCB 4208
140	248	78224	XANTHIOL
145	267	93752	ERGOMETRINE
151	276	186064	MBL-61
152	278	186067	METHYLERGONOVINE
154	280	186061	METHYLSERGIDE
159	304	72138	DESERPIDINE
161	306	169414	MEPIRESERPATE
162	307	169423	METHOSERPIDINE
172	317	15628	RESCINNAMINE
173	318	59272	RESERPINE
175	320	77031	SU 5171
176	321	169861	SU 7064
177	306	169862	SU 9064
180	324	77030	SYROSINGOPINE
182	341	172131	FENOHARMAN
199	374	522806	Cqd 280
215	397	169449	IPRINDOLE
222	408	170993	N-METHYL-1-METHYLTRYPTAMINE
226	418	169107	OXYPERTINE
235	429	107432	SOLYPERTINE
245	501	169869	DIMEFLINE
248	504	169870	REC 7-0105
250	523	115748	CHLORDIAZEPOXIDE
251	528	169897	DIAZEPAM
252	538	169448	OXAZEPAM

APPENDIX 1 (continued)

P No.	2nd. Ed. No.	NSC	NAME
255	526	169898	RO 5-2092
256	540	46078	RO 5-2180
257	541	46079	RO 5-2181
259	543	169895	RO 5-3027
260	536	58775	RO 5-3059
261	534	169896	RO 5-4556
263	601	170980	ACEPERONE
264	656	169410	AF-365
266	658	169409	AF-403
277	680	56321	ARECOLINE
279	684	169427	AZACYCLONOL HYDROCHLORIDE
280	687	58187	BEMEGRIDE
281	608	169873	BENZPERIDOL HYDROCHLORIDE
281	608	170982	BENZPERIDOL
282	688	169193	BENZQUINAMIDE
283	689	169913	BENZTROPINE METHANE SULFONATE
284	690	170950	BIPERIDEN
285	691	25141	BUCLIZINE HYDROCHLORIDE
287	610	170975	BUTROPIPAZONE
288	953	106570	ROLODIN
293	702	169098	CHLORBENZOSAMINE
294	703	169496	CHLORCYCLIZINE HYDROCHLORIDE
296	704	169108	CHLORMETHAZANONE
298	706	172117	C1 384
299	712	172123	COP
300	713	107429	CYCLAZOCINE
301	715	169102	CYCLIZINE
302	716	169452	CYCRIMINE HYDROCHLORIDE
303	718	84973	CYPROLIDOL
305	615	169874	DROPERIDOL
307	726	169109	DICHLORMETHAZANONE
308	727	169418	DIETHADIONE
311	734	170958	DOXAPRAM
314	868	169477	EUCLIDAN
315	749	172121	FENIMIDE
316	750	169876	FENMETRAMIDE
317	616	170981	FLOROPIPAMIDE
318	617	170977	FLUANISONE
320	619	186062	FR-33
323	621	170973	HALOPERIDOL
324	765	169498	HOMOCHLORCYCLIZINE
325	767	169099	HYDROCHLORBENZETHYLAMINE
326	768	169188	HYDROXYZINE
328	776	81850	IPRONIAZID
329	777	169893	ISOCARBOXAZIDE
330	778	9659	ISONIAZID
335	784	172243	JB-840
339	788	172244	JB-884
340	789	172245	JB-890
342	791	172246	JB-8003
345	794	172247	JB-8026

APPENDIX 1 (continued)

P No.	2nd. Ed. No.	NSC	NAME
346	795	172248	JB-8035
347	796	172249	JB-8087
349	798	172250	JB-8140
351	800	172251	JB-8153
364	816	169419	L1743
367	825	169871	LL-195
375	833	169415	M&B 7150
376	834	169189	MECLIZINE
379	840	33383	MER-16
380	841	31289	MER-22
381	842	170959	METAXALONE
382	843	75892	METHAQUALONE
383	844	169918	METHASTYRIDONE
384	623	170974	METHYLPERIDOL
386	848	169868	METHYLPHENIDATE
395	867	124514	NIALAMIDE
397	870	169454	NICOTINIC ACID
399	872	169863	NIKETHAMIDE
401	902	169192	P-2027
402	903	169191	P-2565
403	605	170979	PARAPERIDIDE
404	908	169499	PEMOLINE
405	910	107430	PENTAZOCINE
406	911	66489	PENTYLENETETRAZOL
408	921	405728	PHENMETRAZINE
412	732	172116	PHENYTOIN
413	927	172120	PIPERAZINE PENTANOL
414	928	27162	PIPERIDINE HYDROCHLORIDE
417	931	33379	PIPRADROL HYDROCHLORIDE
419	935	169103	PROCYCLIDINE
420	936	172119	PRODILIDINE
421	937	169914	PROLINTANE
429	944	169894	RO 4-1284
431	850	169889	RO 4-1778/1
432	946	169909	RO 4-1860
433	947	169890	RO 5-0700
434	948	169891	RO 5-0994
435	949	169892	RO 5-1226
436	955	186065	RS-86
445	631	170983	SPIROPERIDOL
446	978	113565	SQ 10,777
448	985	172132	T-134
449	987	169886	TETRABENAZINE
450	988	72108	TETRAHYDROAMINOACRIDINE HYDROCHLORIDE
451	989	74524	TETRAMETHYLSUCCINIMIDE
452	990	66847	THALIDOMIDE
453	991	169509	THIAZENONE
454	992	170962	THOZALINONE

APPENDIX 1 (continued)

P No.	2nd. Ed. No.	NSC	NAME
457	996	169503	TRIMETHADIONE
458	997	62939	TRIOXAZIN(E)
459	633	169875	TRIPERIDOL HYDROCHLORIDE
462	1005	169100	UCB 4268
467	1011	24995	ZOXAZOLAMIDE
468	1042	169422	CLARMIL
470	1048	169568	Go 177
471	1063	26487	MND
474	1066	169426	OXANAMIDE
475	1070	169878	PROMOXOLANE
476	1059	171000	SKF-5
480	1075	77121	ZYLOFURAMINE
482	1094	169879	ALETAMINE HYDROCHLORIDE
485	1097	170999	AMPHETAMINE SULFATE (RACEMIC)
486	1097	27104	D-AMPHETAMINE SULFATE
487	1099	67172	APONEURON
489	1107	169104	1-HYDROXY-(2',5'-DIMETHOXYPHENYL)-2-ISOPROPYLAMINOPROPANE HYDROCHLORIDE
490	1108	76098	CHLORPHENTERMINE
491	1109	169101	Csaq 144
492	1110	522804	Csat 131
498	1122	169105	DOPAMINE
499	1124	170951	L-EPHEDRINE
502	1133	170995	HYDROXYAMPHETAMINE
504	1137	169915	m-HYDROXYNOREPHEDRINE
508	1141	172242	JB-807
510	1144	169447	MEPHENTERMINE
512A	1147	169505	METHAMPHETAMINE (+)
512B		169506	METHAMPHETAMINE (RACEMIC)
515	1151	169916	METHYLDOPA
519	1155	169501	MO-482
520	1158	169106	NOREPINEPHRIN(E)
521	1160	142004	NYLIDRIN HYDROCHLORIDE
523	1164	169446	Ph 481
524	0919	169187	PHENDIMETRAZINE
525	1165	170957	PHENELZINE SULFATE
528	1169	17766	PHENIPRAZINE
530	1172	14029	PHENYLEPHRINE HYDROCHLORIDE
537	1184	170996	SKF-556
538	1185	170956	SYNEPHRINE
541	1192	80664	TRANYLCYPROMINE SULFATE
551	1225	170997	SKF-525A
552	1241	42559	ADIPHENINE
557	1244	33299	DIPHENHYDRAMINE
559	1246	169478	9-FLUORENYLIDENEACETAMIDE
560	1248	169456	LILLY 01780
561	1247	169093	MJ 1986-1
563	1255	169860	PHENYLTOLOXAMINE
567	1277	169179	METHONALIDE
568	1280	169497	PROCAINE
572	1021	106903	ETHOXYBUTAMOXANE

APPENDIX 1 (continued)

P No.	2nd. Ed. No.	NSC	NAME
573	1022	169413	2596IS
584	1317	169411	CENTROPHENOXINE
587	1322	170963	CINTRIAMIDE
589	1329	172122	CYCLOHEXALAMINE
590	1330	169429	CYPENAMINE HYDROCHLORIDE
591	1332	172127	DIAMFEN
594	1335	169428	DIETHYLPROPION(E)
595	1307	169562	EGYT-201
596	1338	169504	ENCYPRATE
597	1128	172254	FENCAMFAMINE HYDROCHLORIDE
599	1344	172118	GUANOXYFEN SULFATE
603		78117	2-INDAMINE
605	1350	23431	JB-835
607	1351	169455	LILLY 51641
612	1356	50788	MEPHENESIN
613	1356	169508	MEPHENESIN CARBAMATE
614	1359	169457	METAGLYCODOL
615	1361	170960	METHOCARBAMOL
617	1368	77097	MR-1230
619	1370	43798	PARGYLINE
620	1371	169451	PHENAGLYCODOL
623	1374	64270	PHENPROBAMATE
625	1376	169887	PIVALYLBENZHYDRAZINE
626	1380	169907	RO 4-1027
627	1381	169908	RO 4-1108
629	1383	169888	RO 5-1162
630	1384	169883	RO 5-1221
633	1391	47996	SQ 10,220
637	1414	169507	DIETHYLPROPANEDIOL
640	1431	172124	CARISOPRODOL
649	1441	169917	EMYLCAMATE
656	1448	172125	METUCHEN
658	1451	172126	TYBAMATE
661	1465	30372	ETHCHLORVYNOL
669	1499	169502	ECTYLUREA
672	1508	169884	RO 2-7687
673	1509	169904	RO 4-1018
674	1510	169885	RO 4-1038/1
675	1511	169905	RO 4-1107
676	1512	169906	RO 4-1340
677	1513	169901	RO 4-1343
678	1514	169902	RO 4-1385
679	1515	169903	RO 4-1679
685	1526	170998	PROPYLHEXEDRIN(E)
688	1546	25953	DISULFIRAM

APPENDIX 2. SUPPLIER INDEX

Abbott Laboratories
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ACF Chemiefarma NV
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Bloomfield, New Jersey 07003

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Philadelphia, Pennsylvania 19101

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Rensselaer, New York 12144

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L. Zambeletti S. P. A.
Stabilimenti Chimico, Farmaceutici
Via Zambeletti
20021 Baranzate
Milano, Italy

APPENDIX 3. NAME VS. P NUMBER INDEX

NAME	P NO.
ACEPERONE	263
ACETOPHENAZINE MALEATE	2
ADIPHENINE	552
AF-365	264
AF-403	266
ALETAMINE HYDROCHLORIDE	482
AMINOPROMAZIN(E) FUMARATE	3
AMITRIPTYLINE HYDROCHLORIDE	87A
AMITRIPTYLINE N-OXIDE HYDROCHLORIDE	87B
AMPHETAMINE SULFATE (RACEMIC)	485
D-AMPHETAMINE SULFATE	486
APONEURON	487
ARECOLINE	277
AZACYCLONOL HYDROCHLORIDE	279
BEMEGRIDE	280
BENZPERIDOL	281B
BENZPERIDOL HYDROCHLORIDE	281A
BENZQUINAMIDE	282
BENZTROPINE METHANE SULFONATE	283
BIPERIDEN	284
BUCLIZINE HYDROCHLORIDE	285
BUTRIPTYLINE	88
BUTROPIPAZONE	287
C1 384	298
Cqd 280	199
Csaq 144	491
Csat 131	492
CARBAMAZEPIN(E)	89
CARISOPRODOL	640
CARPHENAZINE	5
CENTROPHENOXINE	584
CHLORBENZOSAMINE	293
CHLORCYCLIZINE HYDROCHLORIDE	294
CHLORDIAZEPOXIDE	250
CHLORIMIPRAMINE	90
CHLORMETHAZANONE	296
CHLORPHENTERMINE	490
CHLORPROETHAZINE	12
CHLORPROMAZINE	13
CHLORPROMAZINE SULFOXIDE	14
CHLORPROTHIXENE	91
CINTRIAMIDE	587
CLARMIL	468
CLOPENTHIXOL	93
CLOTHIXAMIDE MALEATE	94
COP	299
CYCLAZOCINE	300
CYCLIZINE	301
CYCLOHEXALAMINE	589
CYCRIMINE HYDROCHLORIDE	302
CYPENAMINE HYDROCHLORIDE	590
CYPROHEPTADINE	95
CYPROLIDOL	303
DEPTROPINE CITRATE	99
DESERPIDINE	159
DIAMFEN	591
DIAZEPAM	251
DIBENZEPIN HYDROCHLORIDE	98
DICHLORMETHAZANONE	307
DICHLORPROMAZIN(E)	19
DIETHADIONE	308
DIETHYLPROPANEDIOL	637
DIETHYLPROPION(E)	594
DIMEFLINE	245
DIMETHACRIN(E)	100
DIMETHOXANATE	21
DIPHENHYDRAMINE	557
DISULFIRAM	688
DIXYRAZINE	22
DOPAMINE	498
DOXAPRAM	311
DOXEPIN	101
DROPERIDOL	305
ECTYLUREA	669

APPENDIX 3 (continued)

NAME	P NO.
EGYT-201	595
EMYLCAMATE	649
ENCYPRATE	596
L-EPHEDRINE	499
ERGOMETRINE	145
ETHCHLORVYNOL	661
ETHOPROPAZIN(E)	23
ETHOXYBUTAMOXANE	572
ETHYLISOBUTRAZINE	24
EUCLIDAN	314
FENCAMFAMINE HYDROCHLORIDE	597
FENIMIDE	315
FENMETRAMIDE	316
FENOHARMAN	182
FLOROPIPAMIDE	317
FLUANISONE	318
9-FLUORENYLIDENEACETAMIDE	559
FLUPENTIXOL	104
FLUPHENAZINE DECANOATE	26
FLUPHENAZINE DIHYDROCHLORIDE	27
FR-33	320
Go 177	470
GUANOXYFEN SULFATE	599
HALOPERIDOL	323
HOMOCHLORCYCLIZINE	324
HOMOPHENAZINE	29
HYDROCHLORBENZETHYLAMINE	325
1-HYDROXY-(2',5'-DIMETHOXYPHENYL)-2-ISOPROPYLAMINOPROPANE HYDROCHLORIDE	489
HYDROXYAMPHETAMINE	502
m-HYDROXYNOREPHEDRINE	504
HYDROXYZINE	326
ID 22	108
IMIPRAMINE	109
IMIPRAMINE N-OXIDE	110
2-INDAMINE	603
IPRINDOLE	215
IPRONIAZID	328
2596IS	573
ISOCARBOXAZIDE	329
ISONIAZID	330
ISOTHIPENDYL	111
JB-807	508
JB-835	605
JB-840	335
JB-884	339
JB-890	340
JB-8003	342
JB-8026	345
JB-8035	346
JB-8087	347
JB-8140	349
JB-8153	351
KS-24	31
KS-33	32
KS-75	33
L1743	364
LILLY 01780	560
LILLY 51641	607
LL-195	367
M&B 7150	375
MPL-61	151
MECLIZINE	376
MEPHENESIN	612
MEPHENESIN CARBAMATE	613
MEPHENTERMINE	510
MEPIRESERPATE	161
MER-16	379
MER-22	380
MESORIDAZINE	36
METAGLYCODOL	614
METAXALONE	381
METHAMPHETAMINE (+)	512A
METHAMPHETAMINE (RACEMIC)	512B

APPENDIX 3 (continued)

NAME	P NO.	NAME	P NO.
METHAQUALONE	382	PEMOLINE	404
METHASTYRIDONE	383	PENTAZOCINE	405
METHDILAZIN(E)	37	PENTYLENETETRAZOL	406
METHOCARBAMOL	615	PERPHENAZIN(E)	49
METHONALIDE	567	PERPHENAZINE SULFOXIDE	50
METHOSERPIDINE	162	PHENAGLYCODOL	620
METHOXYPROMAZINE MALEATE	41	PHENDIMETRAZINE	524
N-METHYL-1-METHYLTRYPTAMINE	222	PHENELZINE SULFATE	525
METHYLDOPA	515	PHENETHIAZINE	51
METHYLERGONOVINE	152	PHENIPRAZINE	528
METHYLPERIDOL	384	PHENMETRAZINE	408
METHYLPHENIDATE	386	PHENPROBAMATE	623
METHYLSERGIDE	154	PHENYLEPHRINE HYDROCHLORIDE	530
METUCHEN	656	PHENYLTOLOXAMINE	563
MJ 1986-1	561	PHENYTOIN	412
MJ 1997-1	115	PIPERAZINE PENTANOL	413
MND	471	PIPERIDINE HYDROCHLORIDE	414
MO-482	519	PIPERIDOCHLORPROMAZINE	55
MR-1230	617	PIPRADROL HYDROCHLORIDE	417
NDR A-3680	43	PIVALYLBENZHYDRAZINE	625
NIALAMIDE	395	PROCAINE	568
NICOTINIC ACID	397	PROCYCLIDINE	419
NIKETHAMIDE	399	PRODILIDINE	420
NOREPINEPHRIN(E)	520	PROHEPTATRIENE	123
NORTRIPTYLINE HYDROCHLORIDE	118	PROLINTANE	421
NYLIDRIN HYDROCHLORIDE	521	PROMAZIN(E)	57
OPIPRAMOL	119	PROMETHAZIN(E)	58
OXANAMIDE	474	PROMOXOLANE	475
OXAZEPAM	252	PROPAZEPINE	124
OXYPERPENDYL	120	PROPIOMAZIN(E)	60
OXYPERTINE	226	PROPIOPROMAZINE	61
P-2027	401	PROPYLHEXEDRIN(E)	685
P-2565	402	PROTHIADENE	125
P-5227	121	PROTRIPTYLINE	127
Ph 481	523	REC 7-0105	248
PARAPERIDIDE	403	RESCINNAMINE	172
PARGYLINE	619	RESERPINE	173

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NAME	P NO.
RO 2-7687	672
RO 4-1018	673
RO 4-1027	626
RO 4-1038/1	674
RO 4-1107	675
RO 4-1108	627
RO 4-1284	429
RO 4-1340	676
RO 4-1343	677
RO 4-1385	678
RO 4-1679	679
RO 4-1778/1	431
RO 4-1860	432
RO 4-6011	128
RO 5-0700	433
RO 5-0994	434
RO 5-1162	629
RO 5-1221	630
RO 5-1226	435
RO 5-2092	255
RO 5-2180	256
RO 5-2181	257
RO 5-3027	259
RO 5-3059	260
RO 5-4556	261
ROLODIN	288
RP 3300	64
RP-7360	66
RP 8307	129
RP-9153	67
RP-9260	68
RS-86	436
SKF 10,810	131
SKF 10,812	132
SKF-5	476
SKF-525A	551
SKF-556	537
SKF 5657	70
SOLYPERTINE	235
SPIROPERIDOL	445
SQ 10,220	633
SQ 10,638	133
SQ 10,777	446
SU 5171	175
SU 7064	176
SU 9064	177
SYNEPHRINE	538
SYROSINGOPINE	180
T-134	448
TETRABENAZINE	449
TETRAHYDROAMINOACRIDINE HYDROCHLORIDE	450
TETRAMETHYLSUCCINIMIDE	451
THALIDOMIDE	452
THIAZENONE	453
THIETHYLPERAZINE MALEATE	74
THIOMETHYLPROMAZINE	75
THIOPHENYLPYRIDYLAMIN(E)	134
THIORIDAZIN(E) HYDROCHLORIDE	78
THIOTHIXENE	135
THOZALINONE	454
TRANYLCYPROMINE SULFATE	541
TRIEMPRAZIN(E) TARTRATE	83
TRIFLUOPERAZIN(E)	80
TRIFLUPROMAZIN(E)	81
TRIMEPRAMINE	137
TRIMETHADIONE	457
TRIOXAZIN(E)	458
TRIPERIDOL HYDROCHLORIDE	459
TYBAMATE	658
UCB 2493	84
UCB 4208	138
UCB 4268	462
XANTHIOL	140
ZOXAZOLAMIDE	467
ZYLOFURAMINE	480

APPENDIX 4. NSC NUMBER VS. P NUMBER INDEX

NSC	P NO.	NSC	P NO.	NSC	P NO.
9659	330	80664	541	169409	266
14029	530	81850	328	169410	264
15628	172	84973	303	169411	584
17468	57	93752	145	169413	573
17473	81	100297	100	169414	161
17474	80	106570	288	169415	375
17479	13	106903	572	169418	308
17766	528	106961	134	169419	364
23431	605	107429	300	169422	468
24995	467	107430	405	169423	162
25141	285	107432	235	169424	110
25953	688	108160	101	169426	474
26487	471	108165	135	169427	279
27104	486	111699	133	169428	594
27162	414	113565	446	169429	590
30321	58	115748	250	169431	99
30372	661	124514	395	169446	523
31289	380	142004	521	169447	510
33299	557	169091	37	169448	252
33379	417	169092	115	169449	215
33383	379	169093	561	169450	60
42559	552	169095	22	169451	620
43798	619	169096	84	169452	302
46078	256	169097	138	169453	118
46079	257	169098	293	169454	397
47996	633	169099	325	169455	607
50440	70	169100	462	169456	560
50788	612	169101	491	169457	614
54546	51	169103	419	169466	64
56321	277	169102	301	169467	23
58187	280	169104	489	169468	3
58775	260	169105	498	169469	41
59272	173	169106	520	169470	12
62323	27	169107	226	169471	24
62939	458	169108	296	169472	137
64270	623	169109	307	169473	66
66489	406	169112	98	169474	129
66847	452	169179	567	169475	67
67172	487	169180	2	169476	68
71755	5	169181	49	169477	314
72108	450	169182	50	169478	559
72138	159	169183	21	169496	294
74524	451	169184	88	169497	568
75892	382	169185	93	169498	324
76098	490	169186	111	169499	404
77030	180	169187	524	169500	61
77031	175	169188	326	169501	519
77097	617	169189	376	169502	669
77121	480	169190	121	169503	457
78117	603	169191	402	169504	596
78224	140	169192	401	169505	512A
78714	94	169193	282	169506	512B

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NSC	P NO.
169507	637
169508	613
169509	453
169510	26
169562	595
169568	470
169860	563
169861	176
169862	177
169863	399
169864	89
169865	90
169866	109
169867	119
169868	386
169869	245
169870	248
169871	367
169873	281
169874	305
169875	459
169876	316
169877	43
169878	475
169879	482
169880	29
169881	120
169883	630
169884	672
169885	674
169886	449
169887	625
169888	629
169889	431
169890	433
169891	434
169892	435
169893	329
169894	429
169895	259
169896	261
169897	251
169898	255
169899	91
169900	128
169901	677
169902	678
169903	679
169904	673
169905	675
169906	676
169907	626
169908	627
169909	432
169910	87A
169911	95
169912	127
169913	283
169914	421
169915	504
169916	515
169917	649
169918	383
170950	284
170951	499
170952	104
170956	538
170957	525
170958	311
170959	381
170960	615
170962	454
170963	587
170973	323
170974	384
170975	287
170977	318
170978	459B
170979	403
170980	263
170981	317
170982	281
170983	445
170990	14
170991	83
170992	131
170993	222
170994	132
170995	502
170996	537
170997	551
170998	685
170999	485
171000	476
172116	412
172117	298
172118	599
172119	420
172120	413
172121	315
172122	589
172123	299
172124	640
172125	656
172126	658
172127	591
172128	19
172129	124
172130	125
172131	182
172132	448
172242	508
172243	335
172244	339
172245	340
172246	342
172247	345
172248	346
172249	347
172250	349
172251	351
172254	597
173379	123
176555	87B
186055	55
186056	33
186057	32
186058	31
186059	108
186060	78
186061	154
186062	320
186063	75
186064	151
186065	436
186066	36
186067	152
186068	74
405728	408
522804	492
522806	199

Distribution and Metabolism of 2-Amino-1,3,4-thiadiazole in Mice, Dogs, and Monkeys [1,2,3]

Salah M. El Dareer,* Kathleen F. Tillery, and Donald L. Hill [4]

SUMMARY

The distribution of [5-^{14}C]2-amino-1,3,4-thiadiazole (ATDA) and its metabolites has been studied in mice, dogs, and monkeys. ATDA and its metabolites were separated on thin-layer chromatography and the amounts of these compounds in body fluids and tissues were calculated. In mice injected ip with 100 mg/kg (300 mg/m^2) of ATDA, serum levels decreased logarithmically with a half-life of 2.9 hours; the total amount of metabolites in serum was relatively low at 30 minutes and decreased further through 6 hours. Tissue distribution of ATDA was fairly uniform. In 24 hours, 73% of the total dose was excreted as unchanged ATDA. In dogs injected iv with 3 mg/kg (60 mg/m^2), serum levels decreased linearly, reaching half of the maximum level at 6 hours. The concentration of total metabolites in serum remained nearly constant between 1 and 12 hours. Dogs excreted the unchanged drug through 12 hours and metabolites through 24 hours. For monkeys injected with 3 mg/kg (36 mg/m^2), serum levels decreased linearly, reaching half the maximum value at 13 hours; the serum concentration of metabolites remained almost constant between 1 and 24 hours. In the urine, these monkeys excreted unchanged ATDA through 12 hours and metabolites through 48 hours. The constant rate of loss of ATDA from serum of dogs and monkeys may be due to a process involving glomerular filtration, reabsorption, and active secretion into the proximal tubules by a saturable process. For a monkey given a smaller dose (0.3 mg/kg), the serum levels decreased in an exponential fashion. For a monkey given a large dose (30 mg/kg), an exponential phase preceded the linear phase. The distribution of ATDA in tissues of dogs and monkeys was, to a large extent, uniform. In both animals, however, stomach and bladder contained relatively large amounts.

[Cancer Treat Rep 62:75–83, 1978]

As a cancer chemotherapeutic agent, 2-amino-1,3,4-thiadiazole (ATDA) has an unusual structure and may represent a class of agents having unique biochemical activity. ATDA and its congener, 2-ethylamino-1,3,4-thiadiazole, have antileukemic activity and increase the de novo rate of synthesis of purines in intact animals (1–3). ATDA is also a teratogen in the rat (4). All of these actions are reversed by nicotinamide, indicating that all may result from the same metabolic block. An important site of action for ATDA in L1210 ascites cells is IMP dehydrogenase (5,6), an enzyme involved in purine metabolism. Inhibition of this enzyme is apparently accomplished by a metabolically formed phosphoribosyl derivative of ATDA and, in intact cells, is reversed by nicotinamide (6).

This report concerns the disposition of ATDA and its metabolites in mice, dogs, and monkeys. A preliminary report of this work has appeared (7).

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[2]Supported by contract N01-CM-43780 from the Division of Cancer Treatment (DCT), National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[3]2-Amino-1,3,4-thiadiazole: NSC-4728; CAS reg. No. 4005-51-0. [5-^{14}C]2-amino-1,3,4-thiadiazole was supplied by Dr. J. A. R. Mead, DCT, NCI.

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MATERIALS AND METHODS

Bio-Solv BBS-3 solubilizer was purchased from Beckman Instruments, Palo Alto, Calif, and Liquifluor and Aquasol scintillation solutions were purchased from New England Nuclear Corp, Boston, Mass. All radioactive samples were counted in a liquid scintillation spectrometer equipped with an external standard. Type SA and SG thin-layer sheets were obtained from Gelman Instrument Co, Ann Arbor, Mich. Pellicon membranes fitted into 13-mm stirred ultrafiltration cells were obtained from Millipore Corp, Bedford, Mass. For dogs and monkeys given 3 mg/kg, [^{14}C]ATDA in deionized water was mixed with unlabeled ATDA to give an injection preparation that had a specific activity of 4.2 microcuries/mg. For 0.3 mg/kg the specific activity was 6.8 microcuries/mg and for 30 mg/kg the specific activity was 0.93 microcurie/mg. For injection of mice, the preparation of [^{14}C]ATDA had a specific activity of 1.0 microcurie/mg.

The following thin-layer systems were used to determine the radiochemical purity of [^{14}C]ATDA: Gelman SA sheets and a solvent of chloroform:methyl alcohol (1:1, vol/vol) (system A); Gelman SA sheets and a solvent of toluene:ethyl alcohol (9:1, vol/vol) (system B); Gelman SG sheets and a solvent of benzene:acetone (5:1, vol/vol) (system C); and SG sheets and a solvent of toluene:ethyl alcohol (9:1, vol/vol) (system D). Unlabeled ATDA, detected by ultraviolet light at 254 nm, was used as a reference compound. The solvent front was allowed to migrate to 15 cm. After drying, the sheets were cut into 0.6-cm strips, starting 0.6 cm behind the origin and ending about 1 cm (two strips) beyond the solvent front. The strips were placed in counting vials containing 1 ml of water for 15 minutes before addition of 10 ml of Aquasol. Counting efficiency of these preparations was 79% in a Packard 3330 liquid scintillation spectrometer. Radioactivity migrating with unlabeled ATDA represented the pure compound. ATDA had R_f values of 0.75, 0.25, 0.48, and 0.69 in systems A, B, C, and D respectively and a chemical purity of 99.2%–99.4%.

Animals used in this study were male BDF_1 mice weighing 25–30 g, female beagle dogs weighing 9.6 and 10.1 kg, and female rhesus monkeys weighing between 4.4 and 5.4 kg. All animals were starved overnight, although water was provided. Doses were 100 mg/kg for mice; 3 mg/kg for dogs; and 0.3, 3, or 30 mg/kg for monkeys. Urine from mice, dogs, and monkeys was collected by catheter; blood was collected from the axillary region by exsanguination of mice anesthetized with ether, and from the cephalic, brachial, and saphenous veins of dogs and monkeys. Various organs and tissues from mice, dogs, and monkeys were collected separately, blotted on filter paper, placed immediately on dry ice, and homogenized, after being thawed and weighed, in 4 volumes of deionized water with a Brinkman Polytron equipped with a P-10 generating head (Brinkman Instruments, Westbury, NY). Immediately after homogenization, samples were spotted for thin-layer chromatography. Freezing and storage had no effect on the formation of metabolites. Intestinal contents were removed at the time of tissue collection.

For determination of total radioactivity in biologic samples, 15 ml of Liquifluor containing Bio-Solv BBS-3 (14:1, vol/vol) was added to 0.1 ml of sample and counted. In the measurement of [^{14}C]ATDA and its products, chromatographic system A was used. Portions (2–100 μl) of undiluted serum, urine, tissue homogenates, and standard, unlabeled ATDA were spotted directly onto thin-layer sheets. Migration channels for each radioactive sample were cut into strips and counted as described above. For each channel, the amount of radioactivity at the R_f corresponding to ATDA and the amounts present as each metabolite were calculated.

Binding to serum proteins was determined by filtration of serum containing 100 μg/ml of [^{14}C]ATDA through a Pellicon membrane that allowed passage of molecules of $\leq$ 25,000 daltons under 3.7 atmospheres of nitrogen. Binding to proteins of tissue homogenates was determined by similar filtration of portions of the preparations described above.

RESULTS

In Vitro Experiments

Incubation for 24 hours of [^{14}C]ATDA (100 μg/ml) in mouse, dog, or monkey serum, mouse urine, or mouse liver, kidney, or spleen homogenates revealed that $< 2\%$ of the drug was converted to products other than ATDA. No discernible products appeared in solvent system A. The assay for binding to serum proteins revealed that only 3% of a 100-μg/ml solution of [^{14}C]ATDA was bound to mouse serum proteins, 7% was bound to dog serum proteins, and 4% was bound to monkey serum proteins. Less than 2% of 100-μg/ml solutions became bound to proteins of tissue homogenates.

[^{14}C]ATDA in Mice

Serum levels of ATDA and its metabolites in mice injected ip with 100 mg/kg are displayed in figure 1. The peak serum level of ATDA (101 $\pm$ 2 μg/ml) was observed at 30 minutes, the earliest time of

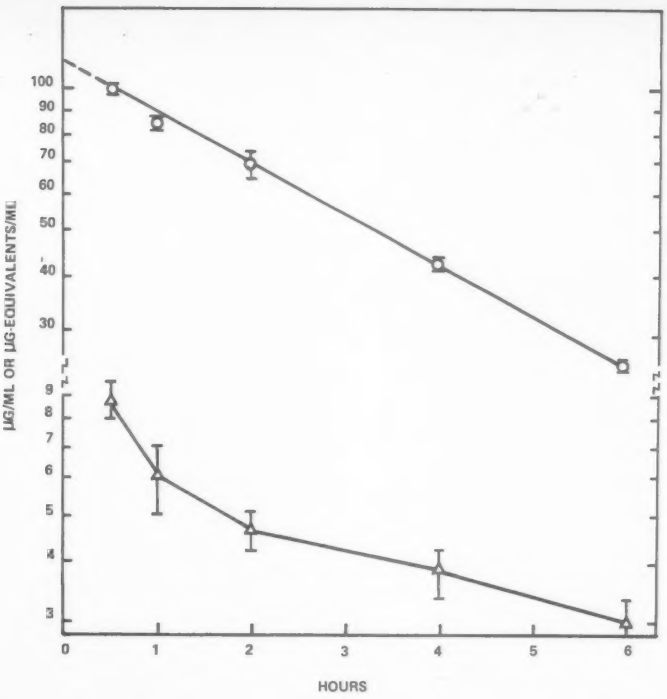

FIGURE 1.—Levels of [^{14}C]ATDA and its metabolites in the serum of mice given an ip dose of 100 mg/kg. Values for the vertical axis are on a logarithmic scale and represent the average ± SD for 3 individual mice. ○ = ATDA; △ = total metabolites.

assay. The concentration in the serum dropped in a logarithmic fashion with an apparent half-life of 2.7 hours. Levels of total metabolites were also highest (9 ± 1 μg/ml) at 30 minutes and fell to 3 ± 0 μg/ml at 6 hours. When this experiment was repeated three additional times (not shown), a half-life of 2.9 ± 0.3 hours was derived. The levels of total metabolites in the following experiments were virtually identical to those for the first experiment. Individual metabolites (I, II, and III, fig 2A) were present in low amounts and in the same relative proportions throughout the experiment. The chromatographic pattern was the same whether or not the serum samples were deproteinized. Levels of [^{14}C]ATDA and its metabolites in the tissues of mice are shown in table 1. The concentration of ATDA in the brain at 30 minutes was less than that in other tissues; after that time, the distribution of ATDA was fairly uniform for all tissues examined. The concentration of metabolites was generally higher in the kidneys, small intestine, spleen, lung, and liver than in other tissues at all times of assay. In the brain, the concentration of metabolites remained low. In a period of 24 hours, three mice excreted 73% of the total dose as ATDA and 22% as metabolites (table 2). During this time the cumulative amounts of ATDA and total metabolites increased in a generally parallel manner. The chromatographic profile of mouse urine is illustrated in figure 2B.

[^{14}C]ATDA in Dogs

Serum levels of ATDA and its metabolites in two dogs given an iv dose of 3 mg/kg are shown in figure 3. At 30 minutes, the serum concentrations were 3.5 and 3.3 μg/ml. For both dogs, the serum concentration of ATDA decreased in an apparently linear fashion throughout the 12-hour period of observation; in 6 hours the amount decreased to one half of its maximum level. The concentration of metabolites in the serum rose for 1 hour, then remained relatively constant throughout the remainder of the experiment. Between the two dogs, there was a difference in that dog B had lower serum levels of ATDA and higher levels of metabolites. The cumulative urinary excretion of ATDA and its metabolites is displayed in figure 4. For both dogs, there was little excretion of unchanged drug after 12 hours, but excretion of metabolites continued

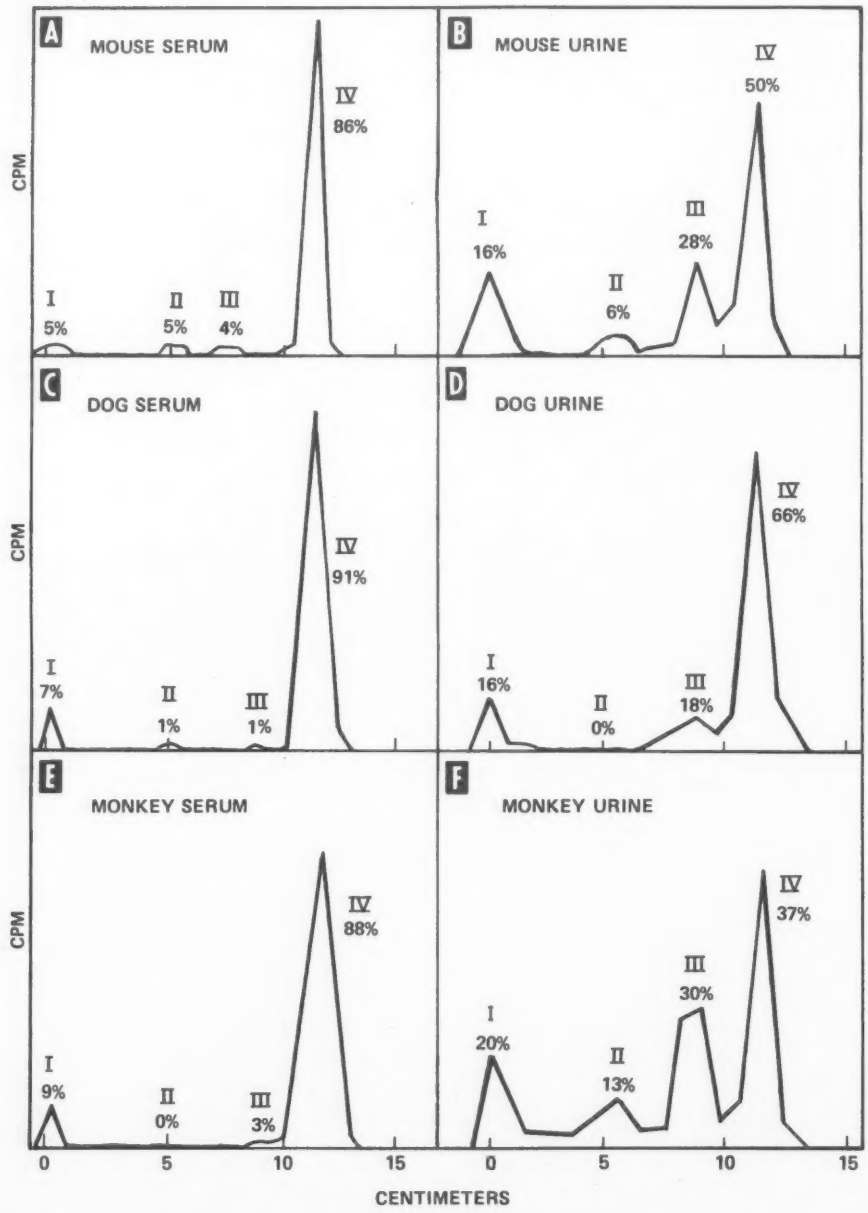

FIGURE 2.—Thin-layer chromatographic profiles of [^{14}C]ATDA and its metabolites after parenteral administration. A and B = mouse serum and urine 4 hrs after an ip dose of 100 mg/kg; C and D = dog serum and urine 2 hrs after an iv dose of 3 mg/kg; E and F = monkey serum and urine 2 hrs after an iv dose of 3 mg/kg. For each chromatogram, the largest peak corresponds to unaltered ATDA. The figures are plotted on a scale indicating the percent of total counts present.

TABLE 1.—Tissue levels of ATDA and its metabolites in mice following ip administration of 100 mg/kg

	μg/g or μg-equivalents/g									
	0.5 hr		1 hr		2 hrs		4 hrs		6 hrs	
Tissue	ATDA	Metabolites	ATDA	Metabolites	ATDA	Metabolites	ATDA	Metabolites	ATDA	Metabolites
Liver	99 ± 5*	8.6 ± 0.4	78 ± 5	7.7 ± 0.5	63 ± 3	6.2 ± 0.3	37 ± 2	7.1 ± 0.4	23 ± 2	5.3 ± 0.4
Kidneys	99 ± 3	14.8 ± 0.5	77 ± 2	11.6 ± 0.4	64 ± 4	9.6 ± 0.6	42 ± 2	5.7 ± 0.3	26 ± 2	5.7 ± 0.5
Spleen	85 ± 2	12.8 ± 0.2	71 ± 4	12.6 ± 0.7	61 ± 1	6.8 ± 0.1	36 ± 2	6.8 ± 0.3	21 ± 2	6.2 ± 0.6
Pancreas†	101	6.5	74	5.5	63	4.0	35	4.8	23	3.8
Lung	92 ± 0	10.2 ± 0.0	75 ± 5	10.2 ± 0.7	61 ± 4	10.0 ± 0.6	36 ± 3	6.8 ± 0.5	22 ± 2	5.2 ± 0.6
Brain	81 ± 4	0.8 ± 0.0	72 ± 3	0.7 ± 0.0	60 ± 1	2.5 ± 0.0	36 ± 2	1.1 ± 0.1	22 ± 2	0.9 ± 0.1
Large intestine	97 ± 2	4.0 ± 0.1	84 ± 4	5.4 ± 0.2	68 ± 5	3.6 ± 0.3	43 ± 4	3.2 ± 0.3	25 ± 2	2.7 ± 0.2
Small intestine	106 ± 2	5.6 ± 0.1	93 ± 8	8.1 ± 0.7	71 ± 5	6.1 ± 0.5	44 ± 5	4.4 ± 0.5	28 ± 0	4.1 ± 0.1
Muscle‡	90 ± 3	1.8 ± 0.1	73 ± 1	3.0 ± 0.0	59 ± 3	2.4 ± 0.1	35 ± 3	1.8 ± 0.2	22 ± 0	1.9 ± 0.2

*Averages ± SD for tissues from 3 individual mice.
†Pooled tissues from 3 mice.
‡Gastrocnemius.

TABLE 2.—Cumulative urinary excretion of ATDA and its metabolites by mice

Collection period (hrs)	% of dose recovered in urine*		
	As ATDA	As metabolites	Total
0–1	12.3 ± 0.6	2.7 ± 0.6	15.0 ± 1.0
0–2	19.0 ± 2.0	8.3 ± 1.2	27.3 ± 1.2
0–4	40.3 ± 1.5	13.3 ± 0.6	53.8 ± 1.2
0–6	45.0 ± 5.7	13.0 ± 1.4	58.0 ± 7.1
0–12	68.7 ± 1.5	19.3 ± 1.5	88.0 ± 3.0
0–24	72.7 ± 0.6	22.0 ± 2.0	94.7 ± 2.1

* Average ± SD for 3 mice.

TABLE 3.—Tissue and fluid levels of ATDA and its metabolites in a dog and a monkey at 4 hrs following an iv dose of 3 mg/kg

Tissue	μg or μg-equivalents/g			
	Dog		Monkey	
	ATDA	Metabolites	ATDA	Metabolites
Bladder	4.84	2.85	2.62	1.67
Kidneys	3.66	1.29	1.84	2.17
Liver	2.03	1.88	1.85	1.08
Spleen	2.60	0.23	0.96	1.56
Pancreas	2.47	0.31	1.47	1.02
Duodenum	2.20	0.17	1.25	0.80
Small intestine	2.38	0.24	1.12	0.74
Large intestine	2.23	0.28	0.92	0.82
Stomach	5.18	0.22	2.72	0.17
Colon	2.49	0.25	2.12	0.18
Lymph nodes	2.60	0.17	2.25	0.22
Adrenal glands	2.10	0.21	2.19	0.14
Submaxillary glands	2.51	0.16	2.32	0.23
Thyroid gland	2.76	0.09	2.24	0.14
Thymus	1.77	0.11	2.28	0.23
Bone marrow	1.75	0.17	1.08	0.12
Voluntary muscle	2.45	0.08	2.41	0.07
Cardiac muscle	2.60	0.14	2.38	0.07
Cerebrum	2.43	0.05	2.21	0.05
Cerebellum	2.38	0.13	2.18	0.11
Lung	2.51	0.22	5.91	0.18
Serum*	2.94	0.44	2.69	0.59
Cerebrospinal fluid*	2.38	0.13	2.45	0.05

* μg or μg-equivalents/ml.

through 24 hours. After 1 hour, there was little change in the relative amounts of serum metabolites (fig 2C), but the relative amount of metabolite III in the urine (fig 2D) increased with time.

In a single dog killed 4 hours after an iv dose of 3 mg/kg, ATDA was widely, and, to a large extent, uniformly distributed in the tissues (table 3). The unchanged drug accumulated moderately in the stomach, bladder, and kidneys, as compared to serum and other tissues. Highest concentrations of metabolites were present in bladder, liver, and kidneys; only these tissues contained amounts greater than serum. In the tissues, < 2% of the total radioactivity was bound to proteins.

[^{14}C]ATDA in Monkeys

Figure 5 shows the serum levels of ATDA and its metabolites in monkeys injected iv with 0.3, 3, or 30 mg/kg of [^{14}C]ATDA. At 30 minutes the serum concentrations of unchanged ATDA were 0.40, 3.7 ± 0.2, and 36 μg/ml respectively. For those monkeys given 3 mg/kg, the serum levels decreased in a linear fashion, reaching one half of the 30-minute value at 13 hours. For a monkey injected with 0.3 mg/kg, a single, exponential phase of elimination was observed. The half-life for this phase was 4 hours. When a dose of 30 mg/kg was injected into a monkey, an initial exponential phase with a half-life

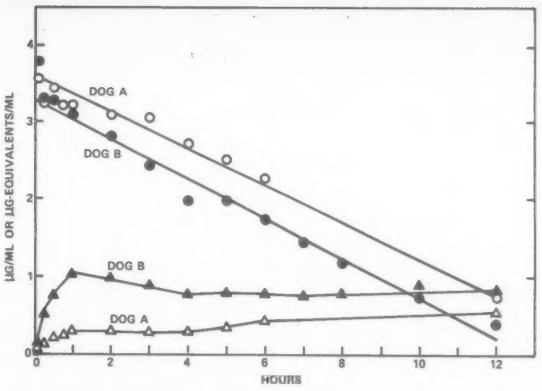

FIGURE 3.—Levels of [^{14}C]ATDA and its metabolites in the serum of 2 dogs given an iv dose of 3 mg/kg. ○, ● = ATDA; △, ▲ = total metabolites.

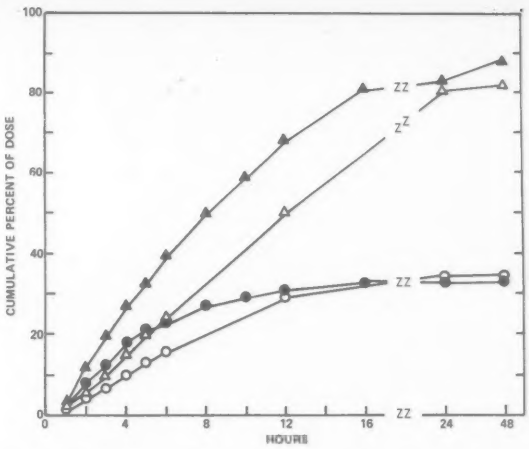

FIGURE 4.—Cumulative urinary excretion of [^{14}C]ATDA and its metabolites by 2 dogs given an iv dose of 3 mg/kg. ○ = ATDA, dog A; ● = ATDA, dog B; △ = ATDA + metabolites, dog A; ▲ = ATDA + metabolites, dog B.

of 10 hours was followed, after 10 hours, by a linear phase.

The concentration of metabolites rose to 0.6 ± 0.1 μg/ml at 2 hours following the 3-mg/kg dose and remained at this level through 24 hours (not shown). For the monkey given 30 mg/kg of ATDA, the serum level of metabolites remained near 1 μg/ml between 2 and 32 hours after injection. At 48 hours the serum concentration of metabolites was 0.16 μg/ml. No metabolites were discernible following the dose of 0.3 mg/ml.

Figure 6 shows the cumulative urinary excretion of ATDA and its metabolites by two monkeys given 3 mg/kg. The amount of ATDA in the urine increased linearly throughout the observation period. In contrast to results for dogs, substantial excretion of unchanged drug occurred after 12 hours. Excretion of metabolites continued through 48 hours. After 1 hour there was little change in the relative amounts of serum metabolites (fig 2E), but amounts of metabolites II and III in the urine (fig 2F) increased with time relative to ATDA (not shown).

The distribution of ATDA in a monkey killed 4 hours after an iv dose of 3 mg/kg is shown in table 3. In general, ATDA was distributed in the same manner as observed for the dog. Three tissues, lung, stomach, and bladder, contained amounts of ATDA greater than that for the serum. Several tissues, including bladder, liver, and kidneys, contained metabolites in amounts greater than serum.

DISCUSSION

Results from this investigation show that after parenteral administration of [^{14}C]ATDA to mice, dogs, and monkeys, levels of unchanged drug in the blood remain high for several hours. For dogs and monkeys, the concentration of metabolites in the blood reaches a plateau after a short time and remains steady for an extended period. For all three species, tissue concentrations of [^{14}C]ATDA and its metabolites generally follow closely levels in the serum. However, the appearance of ATDA in brains of mice does not allow inhibition of growth of ic L1210 leukemia (M. W. Trader and F. M. Schabel, Jr, unpublished results). The retention of ATDA in the blood and tissues of mice is consistent with the prolonged inhibition of DNA synthesis in rat embryos exposed to this agent (8).

The linear rate of excretion of [^{14}C]ATDA by dogs and monkeys given 3 mg/kg is noteworthy. A possible explanation for this observation is that the drug is subject to glomerular filtration, tubular reabsorption, and secretion into the proximal tubules by an active process. Glomerular filtration would be expected, since ATDA does not bind to the serum proteins of dogs and monkeys. If secretion into the proximal tubules involves a process that is saturated at the amounts of ATDA that are present for several hours after injection, removal from the blood would be a linear process. The enzyme(s) metabolizing ATDA is also apparently saturated for several hours; a change in the rate of metabolism would alter the linear rate of removal from the serum.

Following the dose of 0.3 mg/kg to a monkey, the rate of removal was exponential. At such low serum levels the secretion mechanism is apparently no longer saturated. In contrast, the exponential rate

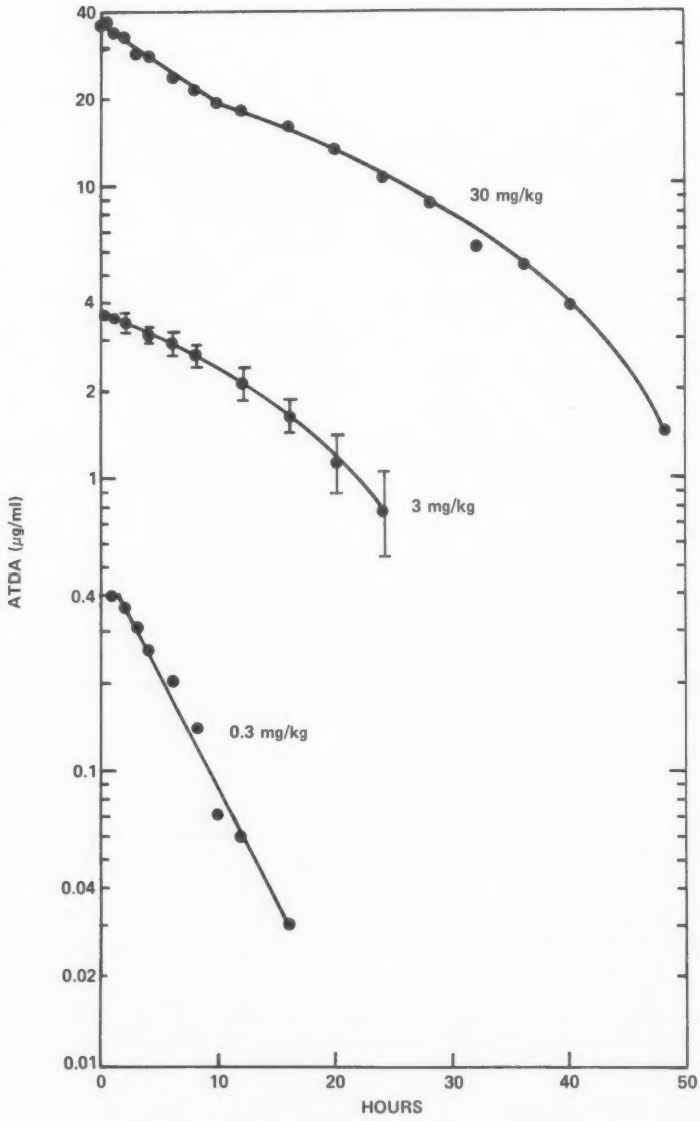

FIGURE 5.—Levels of [^{14}C]ATDA and its metabolites in the serum of monkeys given an iv dose of 0.3, 3, or 30 mg/kg. The values (± SD) for the 3-mg/kg dose are derived from data for 4 monkeys.

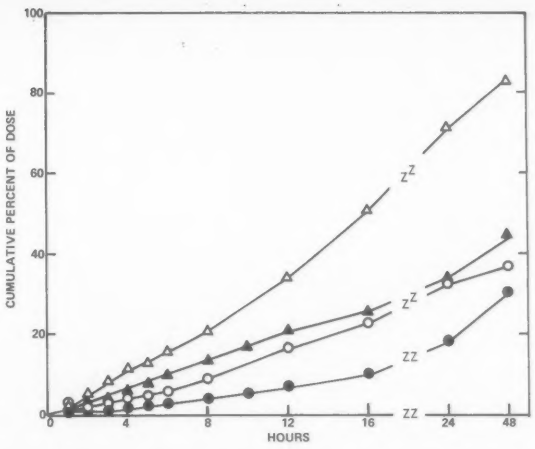

FIGURE 6.—Cumulative urinary excretion of [^{14}C]ATDA and its metabolites by 2 monkeys given an iv dose of 3 mg/kg. ○ = ATDA, monkey A; ● = ATDA, monkey B; △ = ATDA + metabolites, monkey A; ▲ = ATDA + metabolites, monkey B.

observed initially following the 30-mg/kg dose may involve saturation of the tubular reabsorption step; when the levels drop to < 20 μg/ml, this step is no longer saturated.

For mice injected with 100 mg/kg (300 mg/m^2), a dose equivalent to the 30-mg/kg dose for monkeys, the process of removal is logarithmic. This process may be equivalent to the initial, exponential phase observed for the monkey given the high dose.

A preliminary report on the disposition of ATDA in dogs has recently appeared (9). The study also shows that the drug is minimally metabolized. For a relatively large dose of 500 mg/m^2 (approximately 25 mg/kg), an initial plasma half-life of 5 minutes and a terminal half-life of 10 hours were observed. With dogs and monkeys we were unable to determine the half-life of the distribution phase, but our value for the half-life of drug removal from serum of monkeys given an equivalent dose is the same (10 hours) as that reported for dogs (9).

It is likely that the biologic activity of ATDA is related to its conversion to a nucleotide that is present in tissues in very small amounts (6). The data presented here, however, do not allow one to relate tissue concentration of the active metabolite to toxicity in that tissue. In our chromatographic system, nucleotides would probably remain at the origin. Although some material remains at the origin on our chromatograms, it is probably not primarily nucleotides, for these peaks are observed on chromatograms of urine and serum; 5′-nucleotides would probably be dephosphorylated before appearing in these fluids (10). Very likely, the concentration of the ATDA nucleotide in tissues is too low to detect by our procedure. Attempts are being made to identify the major urinary metabolite.

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Chemotherapy of Transplantable Adenocarcinomas of the Colon in Mice. II. Development and Characterization of an Ascitic Line [1,2,3]

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We have previously reported that our MAC series of transplantable colon cancer lines have many characteristics in common with human large bowel cancer (1–3). The histologic appearance of the various tumor lines and their spectrum of chemosensitivity to standard agents make them attractive candidates for pharmacologic research although we recognize that these models would be more demanding as drug screening systems when compared with the standard systems such as the L1210 and P388 mouse leukemias and the B16 melanoma. In these standard systems survival time can be used as an objective endpoint for assessing therapeutic effects; this is not practical with the solid tumor lines in the MAC system. This paper reports the development and characterization of an ascitic form of one of the MAC lines that retains phenotypic properties of the original adenocarcinoma from which it arose.

METHODS

Solid tumor fragments of the two fastest growing lines, MAC 13 and MAC 15, were introduced into the peritoneum of 6–8-week-old NMRI mice by means of a trocar. MAC 13 was inoculated into female mice and MAC 15 into male mice, the solid tumors being routinely passaged in these sexes. After 2 weeks it was apparent that some of the mice that had received tumor fragments of the MAC 15 line had ascites. This was confirmed at laparotomy. The ascites cells were then suspended in TC-199 medium containing penicillin (20 units/ml) and streptomycin (2.0 mg/ml). Aliquots ranging from 0.1 to 1.0 ml were then injected into recipient male mice. For this initial transplantation, no attempt was made to quantitate the inoculum of tumor cells. The recipient mice were observed daily, and after 1 week it was obvious that the mice receiving the largest inoculum had ascites. However, the tumors were allowed to progress for a few days further before an attempt to re-passage the new tumor was made. Preliminary experiments indicated that an inoculum of 10^5–10^6 cells gave the highest incidence of lethal ascites tumors. The line is now routinely passaged using inocula in this range. It has been designated MAC-15A.

CHARACTERIZATION OF THE ASCITES TUMOR LINE

Tumor Growth

After a period of some 3 months covering ten serial passages when consistent survival times in the routine passages were seen and cell count and packed cell volume at the time of transplantation were also stable, the basic growth characteristics of the new tumor were determined. Packed cell volume at the optimum time of transplantation, ie, Day 7 following an inoculum of 10^6 cells, was 20% of the ascitic fluid; the erythrocyte concentration was $< 1\%$.

Figure 1 shows a survival inoculum curve for MAC-15A in NMRI mice. Groups of eight mice were injected ip with $\log_{10}$ dilutions of ascites cells and the median survival time (MST) was calculated as follows: if the total number of animals including survivors (N) is even, the MST (days) = (X + Y + 2), where X is the earliest day when the number of survivors is N/2 and Y is the earliest day when the number of survivors is (N/2) + 1. If N is odd, the MST (days) is X (ref 4). The two sets of points are from experiments 6 months apart illustrating the stability of the new tumor line.

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[3]5-Fluorouracil, BCNU, CCNU, methyl-CCNU, and adriamycin were supplied by the Division of Cancer Treatment, NCI (Bethesda, Md), chlorambucil was supplied by Burroughs Wellcome Ltd, and cyclophosphamide was supplied by Ward Blenkinsop & Co, Ltd.

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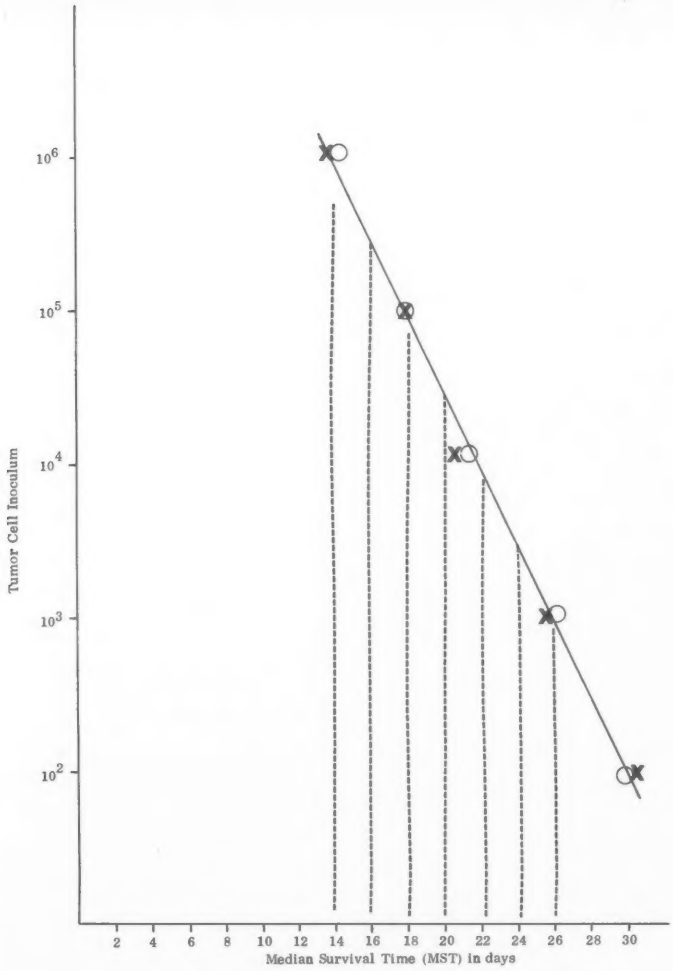

FIGURE 1.—Survival inoculum curve. Serial, $\log_{10}$ dilutions of tumor cells were inoculated into groups of mice, and the MST (days) was measured. The 2 sets of points are from experiments conducted 6 mos apart.

All inocula produced lethal takes, although for calculation of MST it is not necessary to wait for all of the mice to die. The range in survival time varied with the inocula; following 10^6 cells most animals die on the same day, with only an occasional 1- or 2-day discrepancy seen in the odd mouse. This deviation increases with lower inocula, and following 10^2 cells could be as much as 5 days on either side of the MST. From the graph it can be seen that a one-log decrement increases the MST by 4 days, and that the tumor has a doubling time of 1.2 days.

During a 6-month period the survival inoculum curve has been reproduced five times, and on each occasion remarkably similar results were seen; the line drawn represents the best fit to the data from all the experiments, ie, there are 40 animals per point, and therefore the T_d value is reasonably accurate.

Figure 2 shows the results of a ^{3}H-thymidine halving experiment. Ascites cells were labeled in vivo for 1 hour with a pulse of 1.0 microcurie/kg of tritiated thymidine. The cells were washed three times and resuspended in TC-199 containing antibiotics. An aliquot was removed for the determination of the DNA-specific activity, and 0.2-ml aliquots (approximately 2×10^6 cells) were inoculated into 25

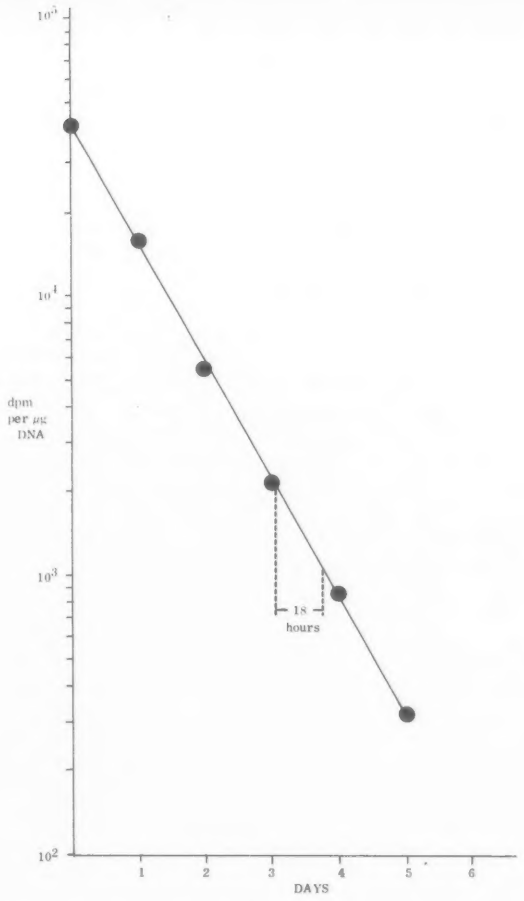

FIGURE 2.—^{3}H-thymidine halving curve. Labeled ascites cells were inoculated into a large group of mice. The DNA-specific activity (dpm/μg of DNA) was followed for 5 days; the specific activity halves, eg, from 2000 to 1000 dpm, over a period of 18 hrs.

mice. Groups of five mice were then killed on Days 1–5 and the tumor cells were washed with saline. The cells were then spun down and the pellet was homogenized in 5.0 ml of distilled water. Duplicate 2.0-ml aliquots were then added to 10.0 ml of ice-cold 0.2 M perchloric acid. The tubes were thoroughly mixed, stoppered, and placed in a refrigerator until all the samples had been collected. Total nucleic acids were extracted by the method of Ogur and Rosen (5). A 0.5-ml aliquot was taken for determination of radioactivity and DNA was measured by the method of Burton (6). Specific activity was expressed as disintegrations per minute per microgram of DNA.

It can be seen that the DNA-specific activity falls by 50% in 18 hours, representing the potential doubling time of the tumor, DT_p; however, from the survival inoculum curve, the actual doubling time, DT_a, is 28.8 hours. According to the calculations of Shirakawa et al (7), then, the cell loss fraction, CL, is given by the formula:

$$CL = 1 - \frac{DT_p}{DT_a} = 1 - \frac{18.0}{28.8} = 1 - 0.625 = 0.375.$$

In other words, the cell division in MAC-15A is 62.5% efficient.

Biologic Characterization

The biology of MAC-15A is probably similar to many other ascites tumor lines. Scanning electron microscope studies have shown that ip inoculation of tumor cells is followed by a rapid attachment to the peritoneal surface. Twenty-four hours after inoculation, large areas of confluent tumor cells are seen. Macroscopic tumor nodules are clearly visible after 1 week, at about the same time as the ascites fluid becomes blood stained. It appears that death is caused by hemorrhage into the peritoneal cavity, and at this point, there are about 10^9 free tumor cells in the cavity and up to ½ g of solid tumor. At this point, many cell aggregates are seen but at the time of transplantation these probably account for < 5% of the total inocula. Sc inoculation of ascites cells produces a solid poorly differentiated adenocarcinoma of similar appearance to the solid deposits in the peritoneum, both of which are less well differentiated when compared with the original solid tumor.

Iv inoculation of the ascites cells produces a variety of systemic tumors depending on the number of cells injected. Low inocula (10^5 cells) mainly produce tumors in the lung, whereas larger inocula (10^6 cells) produce carcinomatosis with tumor nodules in many sites within the thorax and abdomen, and subcutaneously. Preliminary results have shown that there is a reasonable correlation between survival time and tumor load inoculated. Both the low-dose tumor system with multiple pulmonary tumors and the high-dose tumor system with generalized cancer appear to have considerable potential as chemotherapy models. Figure 3 shows a typical poorly differentiated tumor nodule in the lung following iv inoculation of ascites cells.

CHEMOTHERAPY PROTOCOL

In an attempt to produce results that would be comparable to those produced with the solid tumor,

a protocol based on treating "established" ascites tumors was developed. Our electronmicroscopic studies had shown that 24 hours after tumor inoculation the tumor cells were well attached to the peritoneal surface; it was therefore decided that 48 hours after inoculation the tumor would be "established" and suitable for treatment.

A total of 10^6 ascites cells were inoculated ip into large groups of mice on Day 0. On Day 2, ie, 48 hours after inoculation, the mice were randomized into groups of eight. Chemotherapy tests were carried out at three dose levels using a 1.5-fold dose increment; the highest dose was always in the region of the LD10 and often produced deaths from toxicity (*see* table 1). All injections were ip. Solvent controls were always included, and extensions in lifespan were compared with those of the solvent controls. Deaths were recorded daily and the MST was calculated.

In cases where the highest dose was in the region of the LD10 with resultant toxicity, calculations of MST and antitumor activity were omitted. Increase in MST was found from the MST treated (T) minus the MST control (C) (ie, T – C). The percentage increase was calculated as

$$\frac{\text{MST T}}{\text{MST C}} \times 100\%$$

eg, $\frac{12}{10} \times 100 = 120\%$. The percentage cell kill was interpolated from the survival inoculum curve (fig 1) where a one $\log_{10}$ cell kill (90%) equals a 4-day increase in MST.

Chemotherapy Results

Table 1 shows the results of chemotherapy sensitivity studies with seven standard agents, with all except 5-fluorouracil (5-FU) given as single-dose schedules. The most active agent is methyl-CCNU, a single dose of 30 mg/kg giving a 99% cell kill (although 99.5% can be produced by five daily doses of 5-FU). The least active compound of these dose schedules and routes appears to be adriamycin, where at the maximum tolerated dose only a 1-day increase in MST was observed.

TABLE 1.—MAC-15A chemotherapy results*

Drug	Dose schedule	Vehicle	Dose (mg/kg)	Fraction of LD10	No. of toxicity deaths/ No. treated	Increase in MST (days)	% increase in MST (T/C)	% cell kill	MAC 15 % inhibition
5-FU	Single	Saline	200	0.93	0/8	2.5	118	75.0	60†
			133	0.62	0/8	1.5	111	55.0	22
			87.8	0.41	0/8	0.0	100	—	—
	qd × 5		45.0	1.0	0/8	9.0	164	99.5	38
			30.0	0.68	0/8	8.0	157	99.0	—
			20.0	0.46	0/8	4.5	132	93.0	—
Cyclophosphamide	Single	Saline	600	—	2/8	—	—	—	—
			400	0.73	0/8	5.5	139	95.5	86†
			266	0.48	0/8	3.0	121	83.0	76
BCNU	Single	10% ethanol oil‡	90	—	3/8	—	—	—	—
			60	0.86	0/8	2.5	118	75.0	45
			40	0.57	0/8	1.5	111	55.0	20
CCNU	Single	10% ethanol oil	75	—	2/8	—	—	—	—
			50	0.83	0/8	7.0	150	98.3	90†
			33	0.55	0/8	4.0	129	90.0	61
Methyl-CCNU	Single	10% ethanol oil	45	—	2/8	—	—	—	—
			30	0.71	0/8	8.0	157	99.0	66†
			20	0.48	0/8	6.0	143	97.0	55
Adriamycin	Single	Saline	12.0	—	2/8	—	—	—	—
			8.0	0.73	0/8	1.0	107	45.0	24
			5.6	0.48	0/8	2.0	114	70.0	20
Chlorambucil	Single	10% ethanol oil	60	—	3/8	—	—	—	—
			40	0.95	0/8	3.0	121	83.0	55†
			26	0.61	0/8	2.0	114	70.0	50

*In this series of experiments the MST for vehicle controls was 14 days.
†Considered active.
‡Drugs were dissolved in 1/10 vol of dry ethanol and made to volume with dry Arachis oil BP.

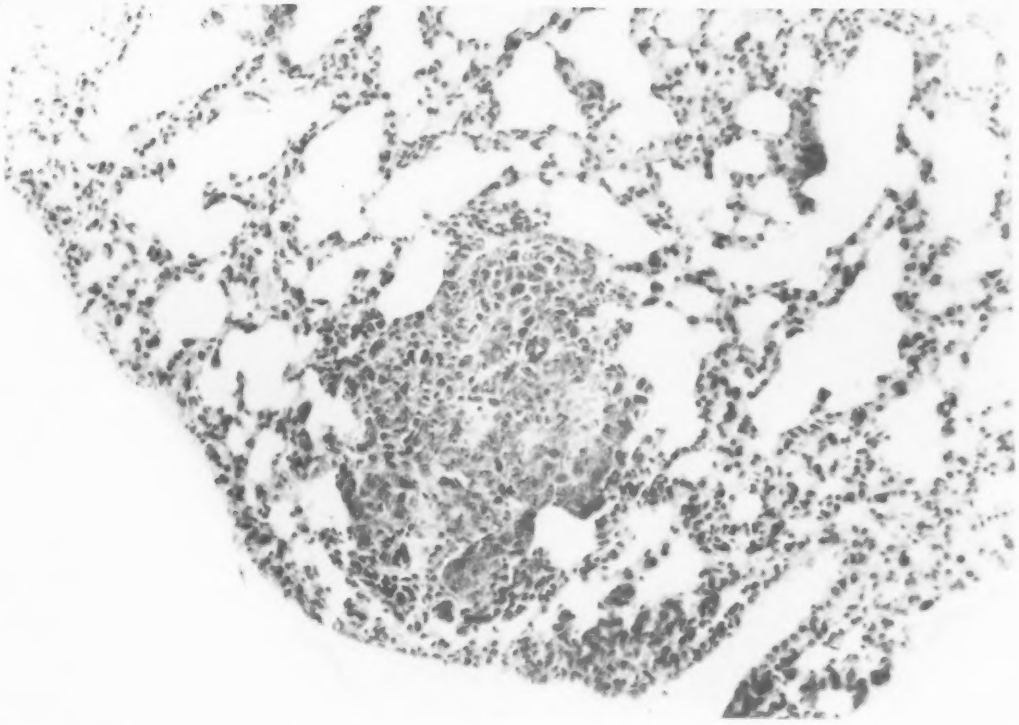

FIGURE 3.—Photomicrograph of a section of the lung from a mouse following iv inoculation of ascites cells. The large nodule of poorly differentiated tumor is clearly seen in the center of the figure (hematoxylin and eosin stain, × 312.5).

Evaluation and Discussion of Results

For an agent to be classified as active in the standard animal tumor screening systems it must produce a T/C of 125% and this must be reproducible (4). Applying this criterion to our test system, only 5-FU given every day × 5 days, cyclophosphamide, CCNU, and methyl-CCNU would be described as active. In our previous publication (3) we decided that in the solid tumor system significant activity was shown only by 5-FU (single dose), cyclophosphamide, CCNU, methyl-CCNU, and chlorambucil, where differences between treated and control were significant by analysis of variance at $0.05 > P > 0.01$.

MAC-15A is obviously a fairly resistant tumor and although a T/C ratio of 125% could be considered a rather low criterion for activity in this type of assay system, the results in table 1 show that agents active in both the original solid MAC 15 and in this system can produce T/C ratios of > 140%.

A comparison of the results in the two test systems is interesting; 5-FU (single dose) considered to be active against the solid tumor would be considered inactive using a T/C of 125% as the minimum criterion for activity against the ascites. However, it can be seen that a 200-mg/kg dose produces a 75% cell kill, which possibly correlates with a 60% inhibition. A more interesting result is seen with the daily schedule of 5-FU, where a 99.5% cell kill can be produced, yet in the solid system this schedule only accounted for a 38% tumor inhibition. This apparent discrepancy between the two test systems can almost certainly be accounted for by the physical differences between them. The solid tumor system MAC 15 used the ip route of drug administration against the solid sc tumor and therefore extra physiologic barriers must be crossed. The ascites (MAC-15A) uses an ip route to an ip tumor. This method produces greater sensitivity, ie, greater antitumor effects are seen, and because of this it has recently been reported that the colon tumor No. 26 from the Southern Research Institute (Birmingham, Ala) has been adapted for ip-ip assay and has been incorporated into the range of tumor used in the secondary screening program by the NCI (8).

The remaining results show a good correlation between the two systems. We are presently extending the range of agents tested against the ascitic line, but the results to date suggest that MAC-15A would be a suitable model system for screening of new agents for potential use in large bowel cancer.

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Metastatic Cloacogenic Carcinoma of the Anus: Sequential Responses to Adriamycin and *cis*-Dichlorodiammineplatinum(II) [1]

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SUMMARY

A case of cloacogenic carcinoma of the anus with pulmonary metastases successfully treated with adriamycin and subsequently with *cis*-dichlorodiammineplatinum(II) (CDP) is reviewed. Cloacogenic carcinoma of the anus is a very uncommon tumor, and this is the first report of a major response to chemotherapy alone. Adriamycin and/or CDP should be considered in the therapy of this tumor.

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Cloacogenic carcinoma of the anus is an uncommon tumor, comprising only 0.6%–3.0% of all anorectal carcinomas (1–4). It is felt to arise from transitional epithelium at the pectinate line between the columnar rectal mucosa and stratified squamous anal mucosa, histologically similar to portions of the urinary bladder (5). Other terminology has been used for this lesion, including basaloid carcinoma, transitional cloacogenic carcinoma, and squamoid carcinoma.

Although several retrospective series have reported on the clinicopathologic features of this tumor (1–4,6–12), there are few reports on the use of chemotherapy in metastatic cloacogenic carcinoma. We report here the successful treatment of such a case with adriamycin followed by an objective response to *cis*-dichlorodiammineplatinum(II) (CDP) upon relapse. To our knowledge, this is the first reported major response to chemotherapy alone in metastatic cloacogenic carcinoma of the anus.

CASE REPORT

Patient 1 was a 75-year-old man who was hospitalized at Fort Carson Army Hospital on January 26, 1975, for the evaluation of an anal stricture and drainage of a presumed rectal abscess. A biopsy showed cloacogenic carcinoma (fig 1). Liver function tests, liver scan, and chest roentgenogram were normal. On February 10, 1975, the patient had abdominoperineal resection. The specimen contained a 2-cm tumor at the level of the anal ducts extending into perirectal fat, and was metastatic to five of five examined perirectal lymph nodes. Resected margins were microscopically free of neoplasm. Several enlarged para-aortic lymph nodes contained cloacogenic carcinoma on biopsy (fig 2). He did well until August 1975, when he developed progressive perineal pain. This was felt to be secondary to residual retroperitoneal and pelvic tumor. Palliative radiation therapy was administered to the perineum at a dose of 5400 rads in 26 treatments over a period of 38 days, beginning on August 6, 1975. Adjuvant bleomycin was administered beginning on August 12, 1975, at a dose of 10 mg iv each week through October 14, 1975; a chest roentgenogram on this date demonstrated widespread nodular pulmonary lesions consistent with metastatic disease (fig 3).

Adriamycin, 100 mg iv every 3 weeks (55 mg/m^2), was instituted on October 28, 1975. A chest roentgenogram on February 2, 1976, demonstrated virtually complete disappearance of the metastatic lesions (fig 4). This therapy was continued until the pulmonary nodules reappeared on June 23, 1976 (total dose of adriamycin, 550 mg/m^2). Treatment was changed to 5-fluorouracil (5-FU), 600 mg iv (350 mg/m^2), and mitomycin C, 25 mg iv (15 mg/m^2) every

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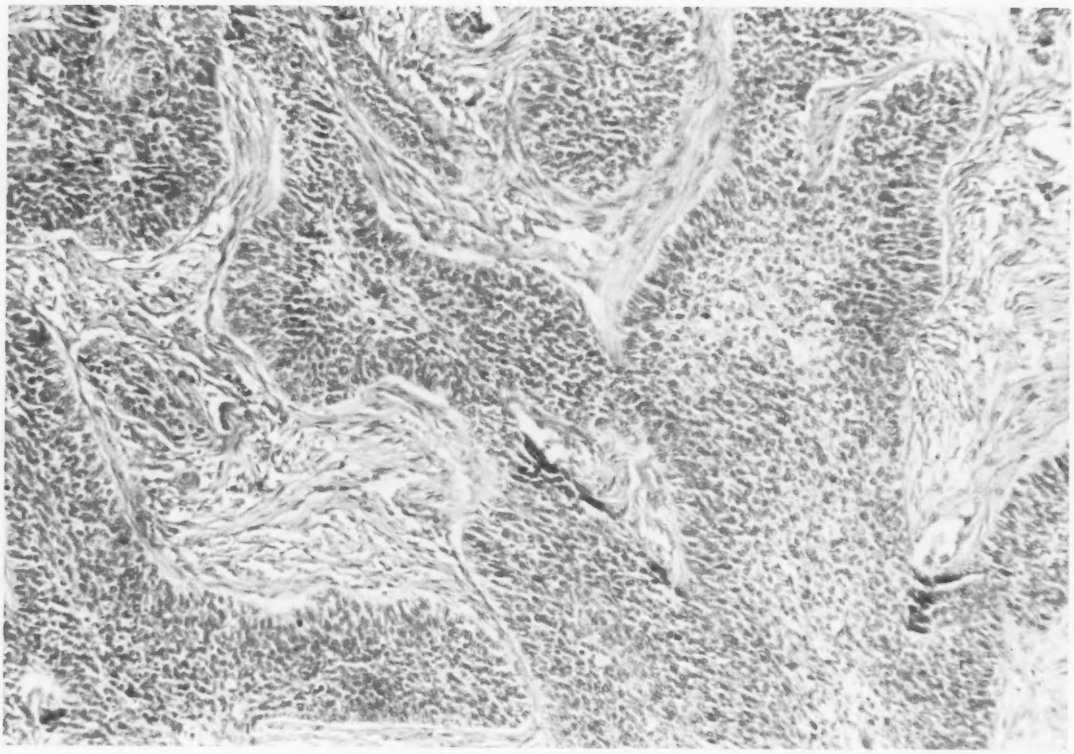

FIGURE 1.—Invasive cloacogenic carcinoma. Note the cords of epithelium resembling that of transitional epithelium lining the primitive urogenital sinus (× 100).

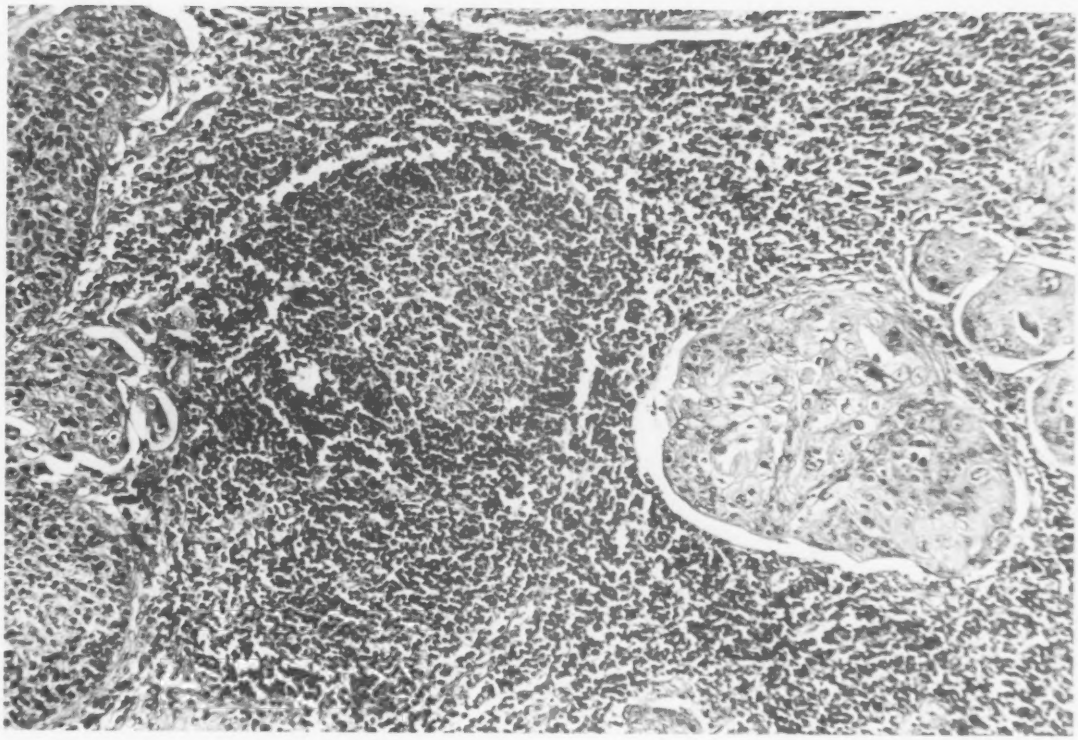

FIGURE 2.—Metastatic cloacogenic carcinoma to para-aortic lymph node. Note the considerable squamous differentiation of the tumor metastasis (× 100).

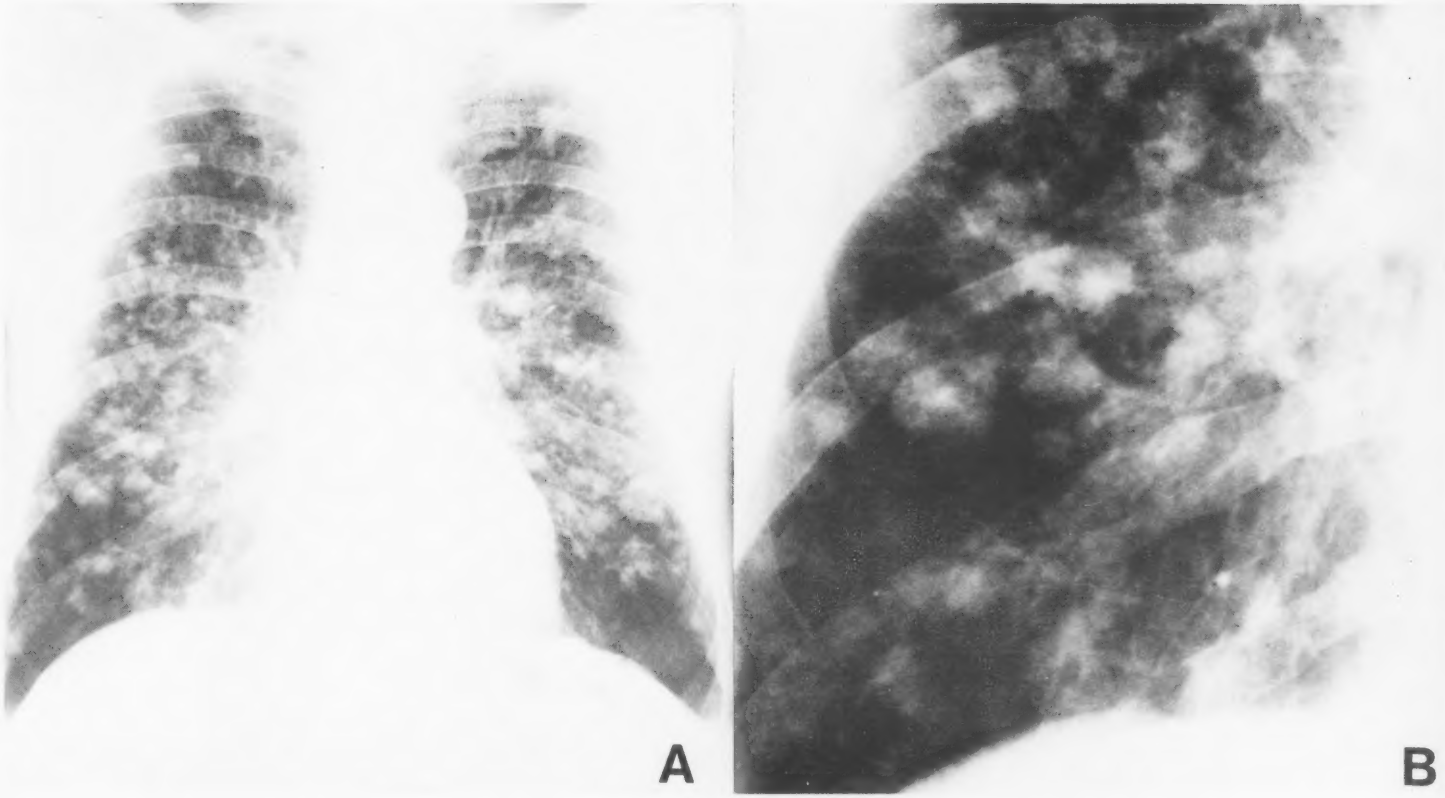

FIGURE 3.—(A) Posteroanterior chest radiograph, Oct 1975, and (B) cone down view of right lung base. These radiographs demonstrate extensive pulmonary nodular metastases with no osseous or cardiac abnormalities.

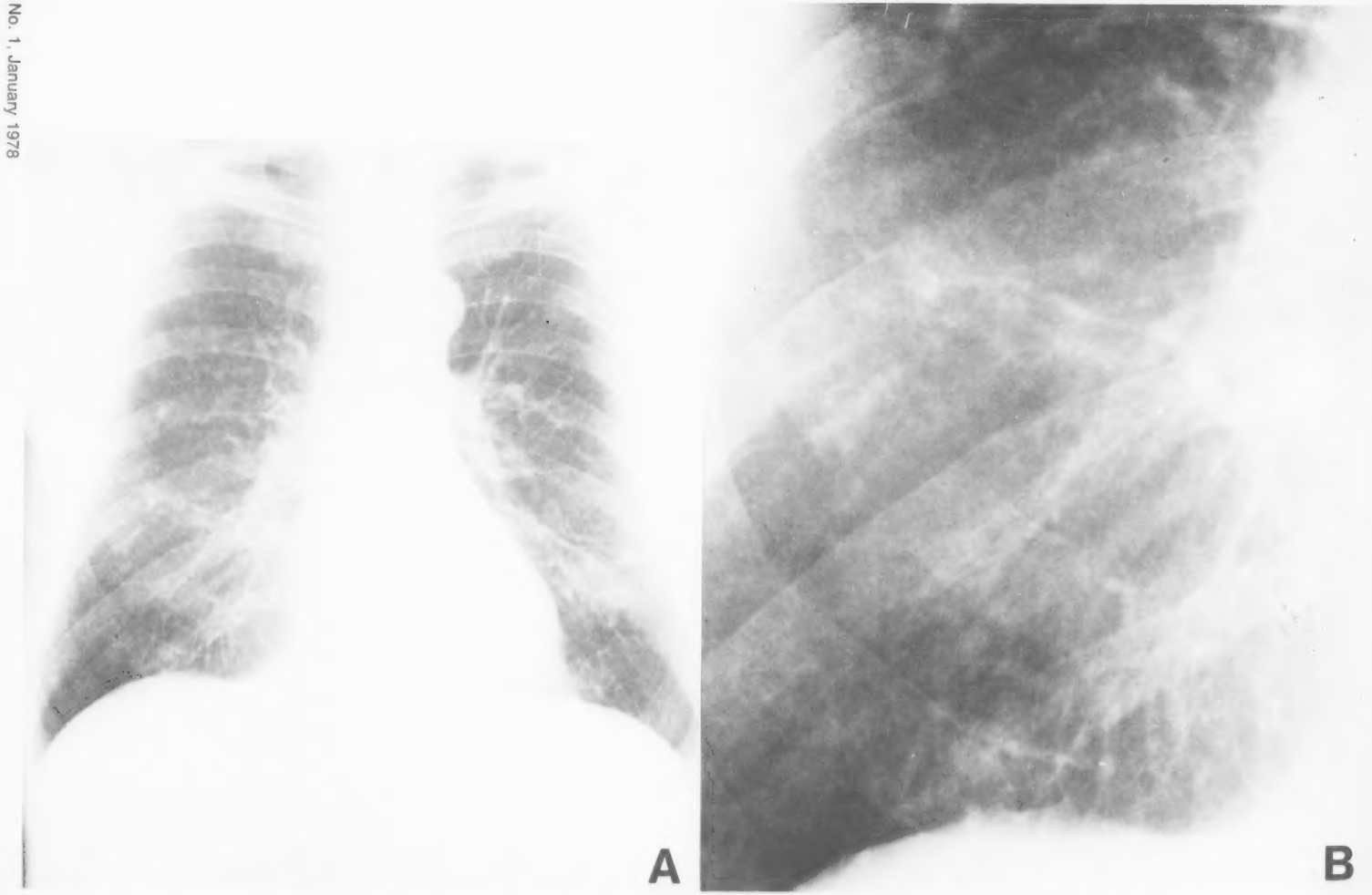

FIGURE 4.—(A) Posteroanterior chest radiograph, Feb 1976, and (B) cone down view of right lung base. These radiographs demonstrate marked decrease in the number and size of the pulmonary metastases.

3 weeks, for two courses. The pulmonary lesions progressed and a right pelvic mass (14 × 12 cm) appeared. He received 4000 rads of radiation to the whole pelvis with a 1000-rad boost to the right hemipelvis over a 5-week period. The pelvic mass decreased in size (6 × 8 cm) but a 1 × 5-cm left supraclavicular node appeared. On December 29, 1976, he received a 6-hour infusion of 1000 ml of D5 ½ NNaCl with 44 mEq of $NaHCO_3$ followed by a 30-minute infusion of 20 g of mannitol. Immediately thereafter, CDP, 2 mg/kg, was administered iv over 30 minutes followed by a second 20-g infusion of mannitol. He received this therapy approximately every 2 weeks. During the next 16 weeks he experienced symptomatic improvement with decreased pain, improved appetite, loss of pelvic fullness, and further shrinkage of the pelvic mass (6 × 8 cm to a barely palpable area estimated at 3 × 3 cm). Most significant were the complete disappearance of the 1 × 5-cm supraclavicular node and the 50%–75% shrinkage of the pulmonary lesions. Serial creatinine values after CDP therapy never exceeded 1.5 mg/100 ml. He refused further treatment, the last dose being given on April 26, 1977, and he died on June 23, 1977. No autopsy was performed.

DISCUSSION

The surgical approach to cloacogenic carcinoma of the anus has been the same as that used for squamous or epidermoid anal carcinoma (11,13–15). Retrospective analysis of surgical therapy has demonstrated 5-year survivals of approximately 50% in patients who had abdominoperineal resections (3,4,12). Local excision of lesions < 5 cm in diameter produced similar 5-year results (4). More recent reports of combined radiation therapy and surgical resection suggest possible improvements in these results (16–18).

Reports on the use of chemotherapy for metastatic cloacogenic carcinoma have been limited (2,12,14,19,20). Of the five cases reported in which 5-FU was used as a single agent, there were no responses (12,19). Livingston et al reported a partial response using bleomycin and vincristine in a patient with cloacogenic carcinoma of the anus (20). Vinblastine (2) and pelvic infusions of mechlorethamine (14) have been ineffective.

Recently, Nigro et al (17) and Newman and Quan (18) have reported on the use of 5-FU and mitomycin C combined with surgery and radiation therapy for the treatment of carcinoma of the anus. These two studies suggest the possible efficacy of combining chemotherapy with radiation therapy and surgical resection for cloacogenic carcinoma of the anus, but the actual contribution of the 5-FU-mitomycin C combination is impossible to discern.

To our knowledge, this is the first report of a major response of metastatic cloacogenic carcinoma of the anus to chemotherapy alone. Adriamycin has been reported to have little efficacy in the treatment of colorectal carcinoma (21). CDP has been similarly ineffective. However, both drugs appear to be active against transitional cell bladder carcinoma (21–23). The 8-month objective response to adriamycin and the impressive objective response to CDP in our patient document their efficacy in cloacogenic anal carcinoma. This report should stimulate the study of combination chemotherapy protocols using adriamycin and CDP in this tumor.

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Pharmaceutical Assessment of Amygdalin (Laetrile) Products [1,2]

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SUMMARY

The National Cancer Institute (NCI) recently acquired a large supply of formulated products of amygdalin manufactured by Cyto Pharma of Mexico, for possible use in a clinical trial in the US. Tablets for oral administration and ampules of the injectable product were obtained. Both forms were extensively analyzed and evaluated by several analytic and pharmaceutical laboratories under contract with the NCI. Analytic test procedures were developed to determine the chemical integrity and quantitative composition of the formulated products. Routine physical and biologic tests were also performed to evaluate the manufacturing quality of both dosage forms. The results indicate that both the oral and injectable forms of amygdalin were substandard by US criteria for manufactured pharmaceutical products. All samples were determined to be chemically subpotent, mislabeled, and of poor manufacturing quality. More than 20 samples of the ampules were found by visual inspection to contain microbial contamination. Other samples were found to be pyrogenic. Based on the results of the testing performed, both tablet and ampule forms of amygdalin manufactured by Cyto Pharma of Mexico are considered unfit for use in man.

[Cancer Treat Rep 62:99–104, 1978]

Amygdalin, commonly known as Laetrile (1), is a cyanogenic glucoside found in the kernels of bitter almonds, apricots, peaches, and plums (2–4). It has been the subject of considerable controversy over the years with regard to its use in cancer treatment (1,3–12).

In light of this controversy, a large supply of formulated products of amygdalin, manufactured by Cyto Pharma of Mexico, was obtained in July 1977 by the NCI for possible use in a clinical trial in the US. The NCI acquisition was arranged in collaboration with representatives of the Food and Drug Administration, the US Customs Service in Washington, DC, and San Diego, and the Assistant US Attorney of the Department of Justice for the Southern District of California. The products were seized by US Customs Service inspectors between December 1973 and December 1975 while being illegally transported into the US. They were stored in their original containers in the US Customs Service security vault and identified by seizure case numbers. Two dosage forms were obtained: an injectable liquid in amber ampules and tablets for oral administration. A tabulation of the ampules and tablets obtained is shown in table 1.

Because of the possible use of the formulated amygdalin products in clinical trials, it was decided that a pharmaceutical assessment would be performed to determine their suitability for use in man. Since manufacturing control numbers were not apparent on the product labeling or packaging, the seizure case numbers were used as a means of identification for product analysis and evaluation.

[1]Received Oct 13, 1977; revised Nov 2, 1977; accepted Nov 11, 1977.

[2]Samples of the ampules and tablets were subjected to standard product evaluation methods of the US pharmaceutical industry by several laboratories and institutions under contracts N01-CM-67109 (Ben Venue Laboratories, Inc, Bedford, Ohio), N01-CM-67103 (Elkins-Sinn, Inc, Cherry Hill, NJ), N01-CM-33722 (Midwest Research Institute, Kansas City, Mo), N01-CM-33723 (Stanford Research Institute, Menlo Park, Calif), and N01-CM-43743 (University of Iowa, Iowa City) from the Division of Cancer Treatment (DCT), National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

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It is thus possible that more than one production batch could be contained in a seizure group.

DESCRIPTION AND LABELING

The ampules from Cyto Pharma are amber in color, have a 10-ml fill volume capacity, and bear preprinted ceramic labels. The ampules in four of the seizure groups have label information as shown in figure 1a. Ampules of the fifth seizure group (76–2810-00001) have different label information as represented in figure 1b. This last group of ampules is thought to be more recently produced based on the more recent seizure date. The ampules in all groups also bear a pine tree logo. The ampule tips are irregular in height and nonuniform at the closure point. This is an indication that automation was not employed in sealing the product.

The tablets are yellow and measure approximately 12.8 mm in diameter and 4 mm in thickness at the center. The Cyto Pharma pine tree logo is inscribed on one side and the number 500 on the other side of each tablet. The tablets appear to be reasonably uniform in shape and color. The largest seizure group of 28,000 tablets (75–2501-00107) was packaged in plastic bags sealed with standard wire staples. Each bag contained 1000 tablets. The remaining seizure groups of tablets were found in plastic bottles of 1000/bottle. The tablets were not labeled.

The labeling of the ampules is unacceptable by US Pharmacopeia standards (ref 13, pages 8, 583). The most critical label deficiency is the lack of manufacturing control codes which are essential for the identification of historical manufacturing data by batch or lot. In addition, the most recent seizure group of ampules (76–2810–00001) has a label change which omits the quantitative amount of the principal ingredient. None of the ampules from any group listed isopropyl alcohol as a component, although it was present in all of the ampules tested.

TABLE 1.—Cyto Pharma products released to NCI

Seizure No.	Seizure date	Amount
	Ampules	
74-2504-10919	12/28/73	700
75-2504-10716	11/09/74	953
76-2505-11569	04/11/75	200
75-2504-11744	05/21/75	856
76-2810-00001	12/12/75	6
Total		2715
	Tablets	
76-2505-11569	04/11/75	1000
75-2504-11744	05/21/75	2000
75-2501-00107	06/24/76	28,000
Total		31,000

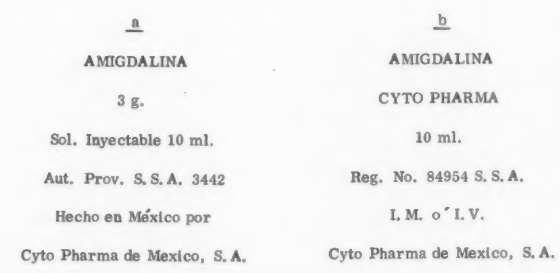

FIGURE 1.—Label information on ampules of Amigdalina: (a) seizure groups 74–2504-10919, 75–2504-10716, 76–2505-11569, 75–2504-11744; (b) seizure group 76–2810-00001.

PRODUCT EVALUATION AND ANALYSIS

Samples of the ampules and tablets were subjected to standard product evaluation methods of the US pharmaceutical industry by several laboratories and institutions under contract to the NCI (*see* footnote 2). (Note: because of the small number of ampules in seizure group 76–2810–00001, only chemical analysis could be performed on this group.) The test results on the ampules and tablets as reported from the various laboratories are summarized in this report.

AMPULES OF AMIGDALINA

Visual Inspection

A 100% visual inspection of the ampule product was made by trained personnel at Elkins-Sinn, Inc. The ampules were washed, evaluated for the presence of an appropriate (10 ml) fill volume, placed under vacuum for the detection of "leakers" (ampules having incomplete seals), and examined carefully under prescribed lighting conditions for particulate matter (14).

Several ampules were found to have low fill volumes. Additionally, several others were discovered to be "leakers." The integrity of the ampule seal is essential for maintenance of sterility. A large number of ampules contained insoluble particulate matter and 20 ampules were found to contain apparent microbial growth. Figure 2 depicts two of the ampules containing this visually apparent contamination. One ampule was found to be unlabeled. A tabulation of the results appears in table 2.

FIGURE 2.—Photograph of visually apparent contamination in 2 Amigdalina ampules.

Pyrogen Testing

The official US Pharmacopeia pyrogen test (ref 13, page 613) for the detection of pyrogenic substances in parenteral products was subcontracted to South Mountain Laboratories of Maplewood, NJ. This test was performed on four of the seizure groups of amygdalin ampules. All ampules tested contained visually clear solutions.

The pyrogen test may be considered positive if one rabbit in three injected rabbits has a temperature rise ≥ 0.6°C, or if the total temperature rise of all three rabbits is > 1.4°C. The results of the pyrogen testing at a dose of 600 mg/kg reveal that pyrogens are present in two of the four groups tested. The fact that one group had no temperature rise of consequence indicates that the drug itself did not interfere with the test and that a pyrogen-free product is obtainable. This test also revealed that at least one group (74–2504–10919) had a very high pyrogen level that could be potentially harmful to man. Although doses of 600 mg/kg have been used, the usual dose is considered to be between 50 and 100 mg/kg (11). Therefore, the pyrogen test was repeated at 50 and 100 mg/kg on the seizure groups positive at 600 mg/kg. In the repeat tests, group 74–2504–10919 was also positive at 100 mg/kg. The 50-mg/kg dose did result in temperature increases in the rabbits, but the magnitude of the response was not sufficient to meet criteria for a positive test result. The results are shown in table 3.

Ben Venue Laboratories also repeated the pyrogen test on seizure group 74–2504–10919 at 50 mg/kg. The results were negative; however, all rabbits had temperature increases (0.5°C, 0.5°C, and 0.2°C).

It was thus ascertained that certain ampules are

TABLE 2.—Summary of visual inspection of Amigdalina ampules

Seizure No.	Total ampules inspected	No. of ampules with— Low fill	Possible leaks	Particulate matter	Apparent growth
74-2504-10919	692	3	5	264	15
75-2504-10716	866	8	2	133	5
76-2505-11569	195	0	0	11	0
75-2504-11744	845	5	1	81	0

TABLE 3.—Results of pyrogen testing

Seizure No.	Maximum rise in temperature (°C)		
	600 mg/kg	100 mg/kg	50 mg/kg
74-2504-10919	2.15	0.95	0.40
	1.65	0.65	0.35
	1.40	1.00	0.10
	5.20*	2.60*	0.85
75-2504-10716	0.55	0.00	0.15
	0.50	0.30	0.00
	0.65	0.15	0.05
	1.70*	0.45	0.20
76-2505-11569	0.50	—	—
	0.30		
	0.05		
	0.85		
75-2504-11744	0.00	—	—
	0.05		
	0.00		
	0.05		

*Pyrogenic at this dose.

high in pyrogenic substances and that pyrogen reactions from some ampules might occur in man at therapeutic dose levels.

In view of the positive pyrogen reaction in rabbits, additional tests were conducted at Ben Venue Laboratories to confirm the presence of endotoxin by the Limulus Amebocyte Lysate method (15,16). This test was originally developed as a quick method for detecting fever-producing endotoxin in radiolabeled products (15). The test is now widely used in the pharmaceutical industry for evaluation of raw materials and certain products that interfere with the official pyrogen test. Ben Venue Laboratories has had considerable experience in this area and, therefore, was considered to be most qualified to perform this test.

A pool of five visually clear ampules from each of four seizure groups was prepared under aseptic conditions in the laboratory. The individual groups were tested at drug concentrations of 300, 30, 3, and 0.3 mg/ml (assuming 3 g/10-ml ampule). The results confirmed the South Mountain Laboratory finding that seizure group 74–2504–10919 contained a high level of endotoxin which was reactive at doses as low as 3 mg/ml. All other seizure groups were negative. The reagent used in the Ben Venue Laboratories test was Pyrogent (Mallinkrodt). Positive control and water controls were performed.

Evaluation of Apparent Microbial Growth

Eight ampules from seizure groups 74–2504–10919 and 75–2504–10716 were evaluated for evidence of microbial contamination at American Type Culture Collection (Rockville, Md). Visually, the samples ranged in clarity from clear to very turbid. In the latter case there was a suggestion of fungal or bacterial growth.

The turbid ampules similar to those shown in figure 2 were found by phase microscopy to contain bipolarly budding yeast and fungal hyphae. The amount of growth present suggests that considerable doubling of cells had occurred but that the resulting environmental changes in the sealed ampules eventually proved to be lethal to the organisms. The organisms appear to be of an air-borne origin associated with dust which may have been introduced during the formulation process. No evidence of bacterial contamination was found by gram staining procedures.

No new growth could be stimulated using standard US Pharmacopeia sterility test methods (ref 13, pages 592–594) in solid and liquid culture media. Thus, it is concluded that no viable organisms presently exist in the product. However, the endotoxin findings and microscopic evidence substantiate the previous existence of viable organisms.

Chemical Identity and Analyses

Analytic methodology for identity and potency of the principal component of both forms of the product was developed by Dr. Peter Lim of the Stanford Research Institute (SRI). The presence of amygdalin was verified by infrared, proton magnetic resonance (PMR), and ultraviolet (UV) spectroscopy. The solution potency was determined by high-pressure liquid chromatography (HPLC). Amygdalin was separated from other UV-absorbing substances using a reverse phase lichrosorb RP8 column and an eluent consisting of aqueous methanol at ambient temperature. Details of this and other analytic methods will be published elsewhere. The ratio of the epimers of amygdalin, D versus L, was determined by PMR as was the isopropyl alcohol content. All ampules tested for chemical content contained visually clear solutions and had fill volumes of 10.0 ± 0.1 ml as an average of six ampules from each seizure group.

The primary component in all five seizure groups of ampules has been verified as D,L-amygdalin in an epimeric ratio of approximately 1:1. The average amount of total intact amygdalin per ampule among the five groups tested is 1.50 g or 50% of the labeled 3-g content. The range of the five groups was 46%–55% of the label. The results of the analysis are summarized in table 4.

The ampules also contain amygdalin-like (UV-absorbing) material. This material has been identified

TABLE 4.—SRI analysis of Amigdalina products

Seizure No.	Amygdalin content by HPLC (%)	D/L ratio	Isopropyl alcohol content by PMR (mg)
	Ampules (3 g)		
74-2504-10919	1.62 g (54)	46/54	49
75-2504-10716	1.66 g (55)	48/52	74
76-2505-11569	1.44 g (48)	49/51	141
75-2504-11744	1.38 g (46)	48/52	150
76-2810-00001	1.42 g (47)	49/51	70
	Tablets (500 mg)		
76-2505-11569	276 mg (55)	All D	—
75-2504-11744	436 mg (87)	All D	—
75-2501-00107	427 mg (85)	All D	—

as the corresponding amide and acid. In addition, isopropyl alcohol was found in the ampules.

Midwest Research Institute (MRI) reported reasonable agreement with SRI in the determination of amygdalin by the HPLC method. The difference between the two analytic laboratories was within 5%. Thus, both analytic laboratories found the ampules tested to be markedly subpotent with respect to amygdalin.

AMIGDALINA TABLETS

Chemical Identity and Analysis

Analytic results (HPLC) from SRI on the three seizure groups of 500-mg tablets revealed an inconsistency in potency ranging from 55% to 87% of intact amygdalin. It should be noted that the tablets contain only the D epimer of amygdalin; compared to the D,L epimeric mixture of the ampules. In addition, two excipients, calcium stearate and starch, have been identified by their infrared spectra. A yellow dye is also present. Other components such as phenobarbital and diazepam were not detected by thin-layer chromatography. Table 4 shows the results of this testing.

Confirmational analyses were performed at MRI and the University of Iowa with similar conclusions of subpotency.

Tablet Disintegration and Dissolution

A final study was assigned to the University of Iowa to perform standard US Pharmacopeia disintegration and dissolution tests on the tablets (ref 13, pages 650–651). A summary of the results appears in table 5.

The pharmacopeias of at least 20 countries set limits of 5–30 minutes as the endpoint for disintegration of ordinary compressed tablets (17). The

TABLE 5.—Disintegration and dissolution of Amigdalina tablets

Seizure No.	Disintegration time (mins)	Dissolution (%) in 30 mins
76-2505-11569	100–120	23.4
76-2504-11744	35–45	37.8
75-2501-00107	25–35	42.0

University of Iowa reports that one of the seizure groups (75–2501–00107) was within acceptable limits. However, two groups (76–2505–11509 and 75–2504–11744) do not meet the test requirements.

The dissolution test is a more sophisticated test for evaluation of tablet quality. This test employs a chemical analysis of dissolved drug in a dissolution medium. The results are plotted as concentration versus time. The US Pharmacopeia has set limits of at least 60% dissolution in 30 minutes in most of the monographs where this test is specified (17). The results of the testing performed indicate group-to-group variation. However, all of the groups fail to meet the generally accepted criteria of the US Pharmacopeia.

CONCLUSION

The testing performed on a large cache of formulated products of amygdalin by Cyto Pharma of Mexico has shown overall poor manufacturing quality with critical deficiencies in a number of areas.

The ampule products did not provide sufficient label information, and the tablets were not labeled at all. All products were determined to be chemically subpotent. Some samples of the ampules from two seizure groups were found to contain fungal contamination. Other samples from these same groups caused pyrogenic reactions in rabbits.

Based on the results of these tests, it is concluded that the products are substandard by usual US

evaluation criteria for manufactured pharmaceutical products and are considered unfit for use in man.

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Antitumor Activity of *N*-trifluoroacetyladriamycin-14-valerate [1]

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SUMMARY

The antitumor activity and toxicity of *N*-trifluoroacetyladriamycin-14-valerate (AD 32) dissolved in 10% Tween 80 in distilled water were compared to those of adriamycin dissolved in distilled water or in 10% Tween 80 in distilled water, in mice bearing L1210 leukemia, P388 leukemia, Gross leukemia, and solid Sarcoma 180. Treatments were performed ip or iv according to different schedules. The antitumor activity of AD 32 was not superior to that of adriamycin. The antitumor activity of adriamycin dissolved in aqueous solution of Tween 80 was higher than that of adriamycin dissolved in distilled water after iv treatment.

[Cancer Treat Rep 62:105–110, 1978]

Considerable attention has been devoted in recent years to investigations on new derivatives of daunorubicin and adriamycin in the hope that modifications of the parent drugs can result in greater antitumor activity and/or decreased toxicity (1–5). Among the several derivatives of adriamycin prepared up to now, *N*-trifluoroacetyladriamycin-14-valerate (AD 32, NSC-246131) was reported to exhibit significantly greater antitumor activity than adriamycin or daunorubicin in several experimental mouse tumor systems (6,7).

In this paper we present data which show that AD 32 is not superior to adriamycin in experimental mouse tumors, and that the selective antitumor activity and toxicity of adriamycin can be strongly modified by administering the compound dissolved in 10% Tween 80.

MATERIALS AND METHODS

Animals

Ha/ICR mice of the CD_1 line, inbred DBA/2 and C3H/He mice, and hybrid (C57BL/6 × DBA/2)F_1 (BDF_1) mice of both sexes were supplied by the Charles River Breeding Laboratories (Calco, Italy). The animals were 2–3 months old, weighed 20–30 g, and were maintained under standard laboratory conditions.

Tumors

Solid Sarcoma 180 (S180) was implanted sc by trocar in the flank of the CD_1 mice. Gross leukemia (8) was transplanted by iv inoculation of 2×10^6 cells of a homogenate of spleens and lymph nodes of leukemic mice in C3H/He mice. Ascitic L1210 leukemia and P388 leukemia, received every 3 months from Mr. I. Wodinsky (Arthur D. Little Inc, Cambridge, Mass) through Dr. F. Spreafico (Mario Negri Institute, Milan, Italy), were maintained by serial passage in DBA/2 mice (according to Geran et al [9]), and transplanted by ip inoculation into BDF_1 mice for experimental purposes (1×10^5 and 1×10^6 cells/mouse, respectively).

Each experimental group consisted of ten animals; in one experiment, some groups consisted of eight mice (due to lack of compound).

Drugs

Adriamycin hydrochloride was supplied by Farmitalia (Milan, Italy). AD 32 was prepared by Dr. F. Arcamone (Farmitalia Research Laboratories, Milan, Italy). Tween 80 furnished by Schuchardt (Munich, Germany) or by Baker Chemical (Deventer, Holland) was used.

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Adriamycin hydrochloride was dissolved in distilled water, and then diluted in distilled water or aqueous solution of Tween 80 at the final concentration of 10%. AD 32 was dissolved in Tween 80 by gentle stirring with a glass rod; the solution so obtained was kept at room temperature overnight, and then diluted in distilled water up to the final concentration of 10% Tween 80. These solutions were administered either ip or iv in a volume of 10 ml/kg of body weight.

The treatment schedules are reported in the Results section. Control groups treated with 10% Tween 80 (10 ml/kg of body weight) were also included.

Evaluation of Antitumor Activity and Toxicity

In solid S180, the antitumor activity was established by the tumor weight index on the 8th and 11th day after tumor implantation. The approximate tumor weight was estimated from tumor diameter measurements, according to Geran et al (9). In all other experimental tumors tested, the comparison of drug effectiveness was based on an increase in median survival time (MST) over the dose range used compared to untreated controls. The number of long-term survivors refers to the surviving mice at the end of the experiments (60 days). Toxicity was established on the basis of the macroscopic necroptic findings evaluated in all dead mice. The main findings were spleen and liver size reduction and hemorrhagic degeneration of the intestinal mucosa, as previously observed after daunorubicin or adriamycin treatment (10).

RESULTS

The antitumor activity of AD 32 was compared to that of adriamycin in ascitic L1210 and P388 leukemias, disseminated Gross leukemia, and solid S180.

In L1210 leukemia-bearing mice treated ip on Days 1–4 (tables 1 and 2), adriamycin dissolved in distilled water showed an optimal dose (giving the highest increase of MST with minimal toxicity: LD10) of 4 mg/kg. At the dose of 6 mg/kg, adriamycin was toxic in 100% of mice in one experiment (table 1) and in 30% of mice in a second experiment (table 2). In this particular experiment at this dose adriamycin also produced an extremely high and unusual increase in lifespan (ILS) (+166%). AD 32 was toxic at the dose of 60 mg/kg; at the LD10 of 40 mg/kg, it was slightly superior to adriamycin given at the dose of 4 mg/kg dissolved in distilled water. Under these experimental conditions, treatment with adriamycin dissolved in 10% Tween 80 resulted in increased toxicity in comparison with similar treatment with adriamycin dissolved in distilled water (table 2).

In P388 leukemia-bearing mice, AD 32 administered ip on Day 1 at the dose of 100 mg/kg (maximal dose tested) exerted the same antitumor effect of adriamycin at the optimal dose of 10 mg/kg (table 3). In this experiment, long-term survivors were not observed, nor were toxic deaths.

In Gross leukemia (table 4) the compounds were administered iv on Days 1–3. The antitumor effect of AD 32 was of the same order of magnitude as that of adriamycin dissolved in distilled water. When adriamycin was administered as a solution in

TABLE 1.—Activity of adriamycin and AD 32 in L1210 leukemia*

Compound	Vehicle	Dose† (mg/kg)	MST‡	ILS§	No. of toxic deaths/No. of mice treated
Untreated controls	—	—	8(8–9)		
Tween-treated controls	—	—	8(7–8)	0	
Adriamycin	H_2O	2	14(10–15)	75	
		4	15.5(8–19)¶	94(1/10)	1/10
		6	7(7–9)	–13	10/10
AD 32	10% Tween 80	10	10(9–10)	25	
		20	12(11–13)	50(1/10)	
		40	18(14–27)¶	125(1/10)	
		60	8(5–8)	0(2/10)	8/10
		80	7(3–9)	–13	10/10

*BDF_1 mice received 10^5 ascites cells ip on Day 0.
†Treatment ip from Days 1 to 4.
‡Days; Nos. in parentheses = range of individual survival times of dead mice.
§Relative to untreated controls (No. of long-term survivors/total No. of mice treated).
¶Not significant (student's t test).

TABLE 2.—Activity of adriamycin and AD 32 in L1210 leukemia, and effect of 10% Tween 80*

Compound	Vehicle	Dose (mg/kg)	MST	ILS	No. of toxic deaths/No. of mice treated
Untreated controls	—	—	9(7–10)		
Tween-treated controls	—	—	9(8–10)	0	
Adriamycin	H_2O	2	15.5(11–21)	72(1/10)	
		4	16(15–30)	77(1/10)	
		6	24(10–60)	166(1/10)	3/10
	10% Tween 80	2	14(8–19)	55	1/10
		4	17(11–22)	89	6/10
		6	13.5(7–17)	50	10/10
AD 32	10% Tween 80	40	18(9–46)	100(1/9)	2/9
		60	11(10–34)	22(3/10)	6/10

*See footnotes to table 1.

TABLE 3.—Activity of adriamycin and AD 32 in P388 leukemia*

Compound	Vehicle	Dose† (mg/kg)	MST‡	ILS§
Untreated controls	—	—	7(7–8)	
Tween-treated controls	—	—	7(7–8)	0
Adriamycin	H_2O	2.5	11(10–12)	57
		5	12(9–13)	71
		10	13(11–16)¶	86
AD 32	10% Tween 80	12.5	8(7–9)	14
		25	9(8–10)	28
		50	10(9–11)	43
		100	13(12–15)¶	86

*BDF_1 mice received 10^6 ascites cells on Day 0.
†Treatment ip on Day 1.
‡Days; Nos. in parentheses = range of individual survival times.
§Relative to untreated controls.
¶Not significant (student's t test).

10% Tween 80, a marked increase in the antitumor activity at nontoxic doses and an increase in the toxicity at the highest dose tested (in one experiment) were observed. In comparison with adriamycin dissolved in 10% Tween 80 therefore, AD 32 was less active in this experimental tumor, at the doses tested. L1210 leukemia-bearing mice were treated iv on Day 1. Results reported in table 5 show that in this experimental system also the administration of adriamycin in 10% Tween 80 resulted in antitumor activity slightly higher than that of adriamycin dissolved in distilled water. AD 32 at doses of 80 and 100 mg/kg was as active as adriamycin dissolved in 10% Tween 80 and administered at doses of 8 and 10 mg/kg.

AD 32 was compared with adriamycin in mice bearing solid S180 treated iv from Days 1 to 5. Results reported in table 6 show that AD 32 caused the same inhibition of tumor growth given by adriamycin at ten times lower doses. Treatment with 10% Tween 80 did not have any effect in the described experimental tumors.

DISCUSSION

In agreement with what has been previously reported by other investigators (6), the data presented in this paper show that AD 32 exerts a significant antitumor activity in mice when it is administered at doses about ten times higher than those of adriamycin. However, under our experimental conditions, the antitumor activity of AD 32 was not superior to that of adriamycin in a number of experimental rodent tumor systems after treatment ip or iv according to different schedules. The techniques employed were essentially the same as those described by Israel et al (6). In particular, we treated L1210 leukemia-bearing mice with the same schedule used by these investigators. The possible

TABLE 4.—Activity of adriamycin and AD 32 in Gross leukemia, and effect of Tween 80*

Compound	Vehicle	Dose† (mg/kg)	MST (days)	ILS‡	No. of toxic deaths/No. of mice treated
Untreated controls	—	—	6.0, 6.0, 6.0		
Tween-treated controls	—	—	6.0, 6.0, 6.0	0, 0, 0	
Adriamycin	H_2O	3.5	8.5	41	
		4.5	10.5, 12.0	75, 100	
		5.5	12.0, 13.0	100, 116	3/20
		6.5	13.5	125	3/10
	10% Tween 80	3.5	15.0, 14.0	150, 133	
		4.5	15.0, 14.0	150, 133	1/20
		5.5	24.5, 16.0	310, 175	2/20
		6.5	10.5	75	9/10
AD 32	10% Tween 80	20	9.0	50	
		30	12.5, 12.0	108, 100	
		40	13.0, 13.0, 13.0	116, 116, 116	3/30
		48	11.5	90	4/10
		57.6	7.0	16	10/10

*C3H/He mice received 2×10^6 leukemic cells iv on Day 0; data of 3 experiments.
†Drugs were administered iv daily for 3 days starting on Day 1.
‡Relative to untreated controls.

TABLE 5.—Effect of iv treatment with adriamycin and AD 32 in L1210 leukemia*

Compound	Vehicle	Dose† (mg/kg)	MST‡	ILS§	No. of long-term survivors¶/ No. of mice treated	No. of toxic deaths/No. of mice treated
Untreated controls	—	—	9.5(9–11)		1/10	
Tween-treated controls	—	—	9(9–11)	-6		
Adriamycin	H_2O	6	11(10–12)	15		
		8	12.5(11–14)	31**	2/10	
		10	12(11–13)	26	1/10	5/10
	10% Tween 80	6	12(12–15)	26		
		8	14.5(13–17)	52**		1/10
		10	14.5(14–17)	52**		
AD 32	10% Tween 80	60	12.5(11–15)	31	1/10	
		80	14(12–15)	47		
		100	14.5(5–19)	52		2/8
		120	15.5(5–27)	63		4/8

*BDF_1 mice received 10^5 ascites cells ip on Day 0.
†Single treatment iv on Day 1 after tumor transplantation.
‡Days; Nos. in parentheses = range of individual survival times of dead mice.
§Relative to untreated controls.
¶At 60 days.
**$P < 0.01$ (student's t test).

TABLE 6.—Activity of adriamycin and AD 32 in solid S180*

Compound	Vehicle	Dose† (mg/kg)	Tumor growth: Day 8 g‡	Day 8 T/C§ (%)	Day 11 g‡	Day 11 T/C§ (%)
Untreated controls	—	—	0.51		1.41	
Adriamycin	H_2O	1.5	0.35	68	0.78	55
		2.0	0.18	32	0.34	24
AD 32	10% Tween 80	15.0	0.31	62	1.13	80
		20.0	0.15	29	0.31	21

*Solid S180 was transplanted into CD_1 mice using trocar pieces.

†Drugs were administered iv daily for 5 consecutive days starting on Day 1 after tumor transplantation.

‡Tumor weight was calculated from measurements of tumor diameter with calipers, according to ref 9.

§Tumor weight of treated mice/tumor weight of control mice × 100.

causes for the differences between our results and the results of these investigators could be the different sensitivity of L1210 leukemia cell lines and of different strains of mice, the different formulation of adriamycin, as Israel et al (6) used clinically formulated adriamycin dissolved in saline while we used purified adriamycin dissolved in distilled water, or the different evaluation of the toxicity of the compounds. In fact, we found that AD 32 was toxic when administered from Days 1 to 4 ip to L1210 leukemia-bearing mice of the BDF_1 strain at the dose of 60 mg/kg, which was described as the optimal one by Israel et al (6).

It was recently reported that AD 32 was more active than adriamycin in a subline of P388 leukemia selected for relative adriamycin resistance (7), did not bind to calf thymus DNA in vitro (11), and differed from adriamycin in the rapid appearance of its fluorescence in the cytoplasm of living cells and the lack of any fluorescent binding to nuclei and chromosomes (12). These data led to the suggestions that either AD 32 is metabolized to a DNA-binding metabolite or its activity is not a consequence of DNA binding. Further studies are necessary in order to elucidate these points.

The modification by Tween 80 of the antitumor activity of adriamycin deserves some comment. We have previously reported the potentiation by Tween 80 of the selective antitumor activity of adriamycin administered iv to Gross leukemia- and solid S180-bearing mice (13). Such an effect was also observed in mice bearing mammary carcinoma and other experimental tumors and in L1210 leukemia-bearing mice treated iv with adriamycin dissolved in 10% Emulphor or 10% Emulphor + 10% ethanol in distilled water, in comparison with adriamycin dissolved in distilled water.[5] These data strongly point to the necessity of carrying out the appropriate controls when Tween 80 or other vehicles are utilized for testing the activity of anthracycline derivatives not soluble in water.

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Comparative Antineoplastic Activity of Adriamycin and *N*-trifluoroacetyladriamycin-14-valerate [1,2]

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SUMMARY

A comparative investigation of the antineoplastic activity of adriamycin and its derivative, *N*-trifluoroacetyladriamycin-14-valerate (AD 32), was conducted in murine tumor models employing different treatment schedules and injection routes. In all conditions tested, ie, ascitic and disseminated L1210 leukemia, ascitic LSTRA lymphoma, and advanced Lewis lung carcinoma, AD 32 was significantly more effective in terms of lifespan prolongation and induction of cures than optimal adriamycin treatments. As with adriamycin, AD 32 was ineffective on ic transplanted L1210 leukemia.

[Cancer Treat Rep 62:111–117, 1978]

In view of its remarkable activity in a wide variety of experimental and human tumors (1–4), much interest has centered in the past few years on adriamycin. A number of efforts have therefore been focused on the search for analogs of this anthracycline antibiotic which possess greater antineoplastic activity and/or reduced toxicity, with special emphasis on a decrease in cardiotoxicity, a limiting side effect in the clinical use of adriamycin (4). Among the analogs so far developed (5–7), *N*-trifluoroacetyladriamycin-14-valerate (AD 32, NSC-246131) appeared of special interest in view of initial reports of a greater antitumor effect in comparison to adriamycin in experimental systems (8). Moreover, this compound may possess at least a partially different mechanism of antineoplastic action (9). It was thus of interest to comparatively evaluate AD 32 and adriamycin employing a larger series of treatment conditions and murine neoplastic models.

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MATERIALS AND METHODS

Animals.—Male mice of the following strains were used: $CD2F_1$, $B6D2F_1$, BALB/c, and C57BL/6, weighing 19–22 g at the start of the experiments and obtained from Charles River Italy (Calco, Italy). Each experimental group consisted of at least ten animals, and gross autopsy was performed on all dead mice to confirm presence of tumor at death.

Tumors.—The L1210 leukemia, provided and renewed every 3 months by Mr. I. Wodinsky (Arthur D. Little Inc, Cambridge, Mass), was maintained by weekly ip passages in DBA/2 mice and transplanted in compatible $CD2F_1$ or $B6D2F_1$ hosts; leukemia cell viability was checked microscopically by dye exclusion and was routinely > 95%. The Moloney virus-induced LSTRA lymphoma was maintained and transplanted in syngeneic BALB/c hosts. The Lewis lung carcinoma (also obtained from Mr. I. Wodinsky) was maintained and transplanted im into C57BL/6 mice, its strain of origin (employing as standard inoculum 10^5 viable cells); spontaneous lung metastases were evaluated as described by Wexler (10).

Drugs.—Three lots of AD 32, one obtained from Dr. F. Arcamone (Farmitalia, Milan, Italy) and two from Drs. M. Israel and E. J. Modest (Sidney Farber Cancer Institute, Boston, Mass), were used after being dissolved in saline-10% Tween 80, strictly adhering to the conditions described by Israel et al (8). Since no differences in chemotherapeutic activity were detected between these lots, results have been pooled. Adriamycin was obtained from Farmi-

talia and was administered in saline unless otherwise specified. Drugs were administered in a volume of 20 ml/kg. Only in the experiments with repeated administration (table 1) were the drugs given in a volume of 10 ml/kg.

Statistical analysis.—Statistical significance of the differences in survival times and percent of long-term survivors was assessed by the Mann-Whitney U test (11) and Fisher's exact test (12), respectively, whereas Duncan's test (13) was employed for the differences in lung metastases weights.

RESULTS

Effect on L1210 Leukemia

A first series of experiments was conducted employing repeated ip injections of adriamycin or AD 32 in $B6D2F_1$ mice transplanted with 10^5 L1210 cells ip on Day 0; the results of four such experiments have been pooled and are shown in table 1. A 4-day treatment beginning on Day 1 with 4 mg/kg of adriamycin resulted in clear increases in lifespan (ILS) but in only occasional > 60-day survivors (10% in two of four tests); an increase in the drug dose to 6 mg/kg was not significantly more active in terms of percent ILS while it resulted in 10%–20% early deaths, ie, before Day 9.

With the same treatment schedule, adriamycin in saline-10% Tween 80 was less effective than adriamycin in saline, the relative decrease in percent ILS values being especially evident at the higher dose level. In the same conditions, AD 32 at 40 or 60 mg/kg was clearly more active than adriamycin; in fact, at both dose levels high and similar percentages of cures were obtained (80%–90% in three experiments and 40% in the fourth test in the case of 60 mg/kg) while no early deaths were seen.

The dose-response curve for the anti-L1210 activity of single iv injections of the two drugs administered on Day 1 is presented in table 2 where one representative experiment of the three which were conducted is shown. It may be seen that the lowest AD 32 dose producing significant lifespan prolongation was 60 mg/kg and that the optimal dose, ie, that which induced the highest percent ILS values without early deaths, was 80–100 mg/kg; in fact, although 120 mg/kg gave the highest ILSs, this dose also consistently resulted in 10%–20% deaths before Day 9, presumably attributable to toxicity. The same table also shows that throughout the active dose range, the percent ILS values found with AD 32 were higher than those seen with adriamycin; in addition, it may be noted that, although > 60-day survivors were seen inconsistently with 80–120 mg/kg of AD 32 (never > 20%), no "cures" were ever seen with adriamycin even at the dose of 15 mg/kg which produced 20%–30% early deaths in these conditions.

The chemotherapeutic effectiveness of these compounds was also evaluated treating L1210-bearing mice at different times after ip tumor transplantation and employing for adriamycin the previously established optimal dose of 10 mg/kg. This adriamycin treatment (table 3) did not produce significant lifespan prolongation when given on Days 5 or 6 and had only borderline activity on Day 4; in contrast, doses of 80 or 120 mg/kg of AD 32 were also significantly active when administered as late as Day 6 and, as judged by the percent ILS values, AD 32 was also more effective than adriamycin with

TABLE 1.—Effect of repeated treatments with AD 32 and adriamycin on ip L1210 leukemia*

Drug (mg/kg)	MST†	% ILS‡	LTS§	Early deaths¶
—	9.2 ± 0.2	—	0/30	—
Tween 80, 10% in saline	8.9 ± 0.2	–3	0/30	—
AD 32 (40)	18.1 ± 2.1	97	14/20**	—
AD 32 (60)	16.7 ± 4.4	81	22/30**	—
Adriamycin (4)	19.1 ± 1.2	108	2/30	—
Adriamycin (6)	18.8 ± 2.1	111	0/30	2/30
Adriamycin (4) in Tween 80	15.1 ± 1.3	70	0/20	3/20
Adriamycin (6) in Tween 80	10.8 ± 1.0	21	0/20	4/20

*10^5 L1210 cells transplanted ip in $B6D2F_1$ mice on Day 0; drugs given ip from Days 1 to 4.
†Mean survival time in days ± 1 SE.
‡% ILS over controls.
§Long-term survivors (> 60 days)/total mice.
¶No. of deaths before Day 9/total mice.
**$P < 0.01$ relative to adriamycin (4 mg/kg) in saline.

injections on Day 3. AD 32 was chemotherapeutically more effective both in terms of prolongation of survival and proportion of cures when tested in mice transplanted iv with 10^3 L1210 cells (table 4); cures were in fact absent at all adriamycin doses tested, whereas 80 mg/kg of AD 32 produced 30%–50% long-term survivors in three experiments.

Because adriamycin does not significantly cross the blood-brain barrier in animals and man (14) and because AD 32 could be expected to be more lipophilic, it was of interest to investigate whether AD 32 exerted an antitumor effect on ic implanted L1210. However, significant prolongations in survival could be observed in none of the four experiments performed, even at drug doses (15 mg/kg iv of adriamycin and 120 mg/kg iv of AD 32) producing early deaths; in the same conditions, 20 mg/kg ip of BCNU, employed as a positive control, gave ILS values of 165%–180%. Also, no significant effects were seen when a 4-day ip treatment with 60 mg/kg of AD 32 or 4 mg/kg of adriamycin was used.

Effect on LSTRA Lymphoma

Table 5 shows the data of one of the three experiments performed in BALB/c mice given single iv doses of the compounds 24 hours after the ip transplantation of 10^5 lymphoma cells. A clear dose-dependent activity was seen for AD 32 in these conditions, the highest ILS values (90%–100%) being found with 100 mg/kg; adriamycin was less effective in the same conditions since the highest dose not producing toxic deaths (10 mg/kg iv) gave ILS values of only 35%–45%.

Effect on Lewis Lung Tumor

Single iv injections of 80 or 100 mg/kg of AD 32 on Day 14 after tumor transplantation, ie, when the primary tumor weight was in the 2.0–2.2-g range, produced significant ILSs over untreated controls with values equivalent to those seen with 15 mg/kg of adriamycin, a dose which was however followed by 30%–40% toxic deaths, ie, before Day 20 (table 6). The well-tolerated 10-mg/kg adriamycin dose was, on the contrary, incapable of significantly prolonging survival in the two experiments performed, in accordance with previous extensive experience in this model system. The effect on pulmonary metastases in the same conditions was examined in two further experiments; the reduction in their total weight seen with 80 mg/kg of AD 32 (89% from control values) was greater than that found with 10 mg/kg of adriamycin (74%), but the difference was on the borderline of statistical significance. It may also be noted that whereas the percentage of adriamycin-

TABLE 2.—Effect of single injections of AD 32 and adriamycin on ip L1210 leukemia*

Drug (mg/kg)	MST†	% ILS	Early deaths‡
—	9.2 ± 0.5	—	—
Tween 80, 10% in saline	9.1 ± 0.6	0	—
AD 32 (40)	10.4 ± 0.7	13	—
AD 32 (60)	12.0 ± 0.6	30	—
AD 32 (70)	13.8 ± 0.5	50	—
AD 32 (80)	14.0 ± 0.5	52	—
AD 32 (90)	15.8 ± 0.4§	72	—
AD 32 (100)	15.3 ± 1.0§	66	—
AD 32 (120)	21.2 ± 2.9§	130	1/10
Adriamycin (6)	10.8 ± 0.3	17	—
Adriamycin (8)	12.0 ± 0.5	30	—
Adriamycin (10)	13.2 ± 0.4	43	—
Adriamycin (6) in Tween 80	11.5 ± 0.4	25	—
Adriamycin (10) in Tween 80	12.0 ± 0.9	30	1/10

*10^5 L1210 cells transplanted ip in B6D2F$_1$ mice on Day 0; drugs given iv on Day 1.

†Mean survival time in days ± 1 SE.

‡No. of deaths before Day 9/total mice.

§$P < 0.05$ relative to adriamycin (10 mg/kg).

TABLE 3.—Effect of single injections of AD 32 and adriamycin on "late" ip L1210 leukemia*

Drug (mg/kg)	Day of treatment	MST†	% ILS
—	—	8.9 ± 0.3	—
AD 32 (80)	3	15.5 ± 0.6‡	74
AD 32 (120)	3	16.8 ± 1.0‡	89
Adriamycin (10)	3	14.0 ± 1.3	57
AD 32 (80)	4	14.5 ± 0.2§	63
AD 32 (120)	4	16.0 ± 0.4§	80
Adriamycin (10)	4	10.8 ± 0.5	21
AD 32 (80)	5	12.7 ± 0.4§	43
AD 32 (120)	5	13.3 ± 0.4§	49
Adriamycin (10)	5	10.5 ± 0.3	18
AD 32 (80)	6	12.5 ± 0.4‡	38
AD 32 (120)	6	13.3 ± 0.5‡	49
Adriamycin (10)	6	11.0 ± 0.2	24

*10^5 L1210 cells transplanted ip in $CD2F_1$ mice; drugs given iv.
†Mean survival time in days ± 1 SE.
‡$P < 0.05$ relative to adriamycin treatment.
§$P < 0.01$ relative to adriamycin treatment.

TABLE 4.—Effect of single injections of AD 32 and adriamycin on iv L1210 leukemia*

Drug (mg/kg)	MST†	% ILS	LTS‡	Early deaths§
—	10.0 ± 0.4	—	0/10	—
Tween 80, 10% in saline	10.4 ± 0.3	4	0/10	—
AD 32 (60)	19.0 ± 2.5¶	90	2/10	—
AD 32 (80)	26.0 ± 6.5¶	160	5/10**	—
AD 32 (100)	39.3 ± 8.9¶	293	2/10	1/10
AD 32 (120)	12.6 ± 2.1	26	3/10	2/10
Adriamycin (5)	11.2 ± 0.4	12	0/10	—
Adriamycin (7.5)	11.6 ± 0.3	16	0/10	—
Adriamycin (10)	12.1 ± 0.2	21	0/10	—

*10^3 L1210 cells transplanted iv in $CD2F_1$ mice on Day 0; drugs given iv on Day 1.
†Mean survival time in days ± 1 SE.
‡Long-term survivors (> 60 days).
§No. of deaths before Day 10/total mice.
¶$P < 0.01$ relative to adriamycin (10 mg/kg).
**$P < 0.05$ relative to adriamycin (10 mg/kg).

treated mice with lung nodules was 100% in both experiments, AD 32 produced 20% and 40% metastasis-free animals at sacrifice on Day 23.

DISCUSSION

The results of this study confirm the conclusion originally advanced by Israel et al (8), employing a more limited number of tumor models and treatment conditions, that AD 32 possesses a better antineoplastic activity than its parent compound adriamycin. This significantly greater effectiveness was seen in the L1210 model not only employing a repeated ip treatment schedule which gave up to 80%–90% long-term survivors but also with single iv injections, and with both types of treatment, the optimal AD 32 dose was eight to ten times higher in weight than that for adriamycin, in agreement with what has been described by others (8). Especially clear in showing the antitumor differential of the two agents after single treatments were the findings obtained in mice with late ip L1210 and those seen in mice transplanted iv with these leukemia cells, a condition in which, at variance to adriamycin, a high proportion of long-term survivors could

TABLE 5.—Effect of single injections of AD 32 and adriamycin on ip LSTRA lymphoma*

Drug (mg/kg)	MST†	% ILS	Early deaths‡
—	9.4 ± 0.2	—	—
Tween 80, 10% in saline	9.0 ± 0.2	-4	—
AD 32 (40)	13.4 ± 0.4	42	—
AD 32 (60)	14.2 ± 0.6	51	—
AD 32 (80)	16.9 ± 0.4§	80	—
AD 32 (100)	17.9 ± 0.3§	90	—
AD 32 (120)	12.4 ± 1.3	32	1/10
Adriamycin (10)	13.2 ± 0.6	40	—

*10^5 LSTRA cells transplanted ip in BALB/c mice on Day 0; drugs given iv on Day 1.
†Mean survival time in days ± 1 SE.
‡No. of deaths before Day 9/total mice.
§$P < 0.01$ relative to adriamycin.

TABLE 6.—Effect of single injections of AD 32 and adriamycin on Lewis lung tumor*

Drug (mg/kg)	MST†	% ILS	Early deaths‡
—	28	—	—
Tween 80, 10% in saline	30	7	—
AD 32 (80)	38§	36	—
AD 32 (100)	39§	39	1/14
Adriamycin (10)	30	7	2/14
Adriamycin (15)	36	30	5/14

*10^5 Lewis lung tumor cells transplanted im in C57BL/6 mice on Day 0; drugs given on Day 14.
†Median survival time.
‡No. of deaths before Day 20/total mice.
§$P < 0.05$ relative to adriamycin (10 mg/kg).

be observed with well-tolerated doses of AD 32. A better antineoplastic activity was also found in the virally induced LSTRA lymphoma and in the Lewis lung carcinoma, a tumor known to be relatively resistant to many chemotherapeutic agents (15), and in which single-drug doses produced significant lifespan prolongation even when administered at an advanced disease stage, a result obtainable with adriamycin only at clearly toxic doses. This demonstration of effectiveness is seen also on solid neoplasms, in agreement with other data (16,17); this is an encouraging sign for possible clinical utilization of this compound. Conversely, there was a lack of effectiveness of otherwise active AD 32 treatments on ic implanted L1210, a condition in which adriamycin was also inactive. The tentative conclusion that AD 32 does not significantly cross the blood-brain barrier is supported by our preliminary pharmacokinetic studies in which no drug could be detected in the brain at times when peak levels were present in blood and/or tissues after the iv injection of 120 mg/kg (unpublished data). The same conclusion would also seem to apply for the biotransformation products of this agent, some of which (eg, *N*-trifluoroacetyladriamycin [AD 41] and 13-dihydro-AD 32 [AD 60]) have been reported to be cytotoxic (18).

In each of the systems investigated, the higher antineoplastic activity of AD 32 with respect to adriamycin was seen after apparently equitoxic treatments as judged by the crude parameter of absence of early, nontumorous deaths. Thus, the better activity of the former agent in systems such as the LSTRA lymphoma and Lewis lung carcinoma

could have been obtained at the expense of greater toxicity not differentiated from tumor-induced death in these conditions. Cardiotoxicity, for instance, a limiting side effect with anthracycline antibiotics (19), can be of delayed appearance in rodents (19,20) and the relative cardiotoxic potential of AD 32 and adriamycin has not yet been established. It is noteworthy however, that high proportions of > 60-day (or longer) survivors could consistently be obtained with AD 32 in two of our experimental systems. Although no comparative evaluation of the toxicity of the two agents was conducted in this study, this observation would suggest that even if the toxicity induced by optimal AD 32 doses is higher than that seen with optimal adriamycin doses, it is in any case compatible with an extended and grossly normal survival in this species.

In supporting a superior effectiveness of AD 32 over adriamycin after single or repeated treatments in a series of murine neoplasms of different origin, growth rate, and responsiveness to chemotherapy, our results are at variance with those recently reported by Pratesi et al (16). These investigators found in fact that amounts of AD 32 ten times higher in weight were essentially as active as optimal doses of adriamycin in 10% Tween 80 which in turn was frequently more active than adriamycin in water when tested in ascitic P388 and L1210 leukemias, disseminated Gross lymphoma, and Sarcoma 180. In addition to producing virtually no cures even after repeated injections, AD 32 was also described as being more toxic than adriamycin in some of their systems at doses resulting in comparable therapeutic effects, although the parameters employed for assessment of drug toxicity (eg, spleen and liver weights at necropsy of tumor-bearing mice) are open to criticism as to their specificity.

Since the L1210 leukemia employed in our study was as sensitive to adriamycin as the subline employed by Pratesi et al (16), the reasons for this discrepancy in results are at the moment unclear. The basis for the superiority of AD 32 in our study is still under investigation. Because adriamycin in Tween 80 was not more effective than when dissolved in water, and because in both vehicles optimal doses of this drug were significantly less active than AD 32, a vehicle effect seems unlikely as a major mechanism in the differential antineoplastic capacity of the two compounds. That AD 32 is not simply a prodrug form of adriamycin is indicated by metabolic studies which have failed to detect significant amounts of adriamycin in AD 32-treated animals (21,22). Evidence has however been obtained that AD 32 may possess a mechanism of action at least partially different from that of adriamycin as suggested by its different intracellular distribution and absence of binding to DNA in vitro (9).

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N-trifluoroacetyladriamycin-14-valerate: Additional Mouse Antitumor and Toxicity Studies[1,2]

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SUMMARY

Recent studies have shown that the therapeutic superiority of *N*-trifluoroacetyladriamycin-14-valerate (AD 32) over adriamycin (ADR) in the murine L1210 leukemia system, as previously reported by us, is not the result of vehicle effect: ADR at optimal doses gave the same prolongation of survival whether formulated in saline or surfactant-containing medium, while AD 32 in an Emulphor-ethanol-saline vehicle gave as high a percentage of "cures" as when administered in 10% aqueous Tween 80. With respect to toxicity, four daily doses of 4.0 mg/kg of ADR in normal BDF_1 mice resulted in toxic deaths (median survival, 21 days), while this same dose is often found by us to be the "optimal" ADR dose in L1210 therapy trials. Thus, in the L1210 system, the maximum activity of ADR appears to represent a compromise between antitumor efficacy and toxicity. In contrast, acute toxic deaths were not seen in normal mice with 20–90 mg/kg of AD 32 for 4 days, a range which includes the optimal dose for AD 32 in the L1210 system. AD 32 is superior to ADR when evaluated against a P388 subline selected for ADR resistance, and it is also active against the solid Ridgway osteogenic sarcoma. Activity in the latter system further demonstrates the ability of this agent to act upon localized tumor at sites distant from the point of drug administration. Both agents produced similar effects on DNA synthesis in normal and tumor tissue, as measured by ^{3}H-thymidine incorporation. This last observation is of interest in view of evidence, presented elsewhere, that AD 32 essentially does not bind to DNA and that ADR is not a significant in vivo metabolite of AD 32.

[Cancer Treat Rep 62:119–127, 1978]

The anthracycline antibiotic adriamycin (ADR) is of major clinical importance in the treatment of leukemias and in the management of a variety of otherwise drug-refractory solid tumors (1,2). Its use, however, is complicated by two dose-limiting toxic effects: acute myelosuppression and cumulative dose-dependent delayed cardiomyopathy (3). Numerous structural analogs have been prepared by semisynthetic modification and de novo synthesis in an attempt to extend the antitumor activity of ADR or to reduce its toxicity (4). From our own program has emerged *N*-trifluoroacetyladriamycin-14-valerate (AD 32, NSC-246131), a less toxic analog with clear therapeutic superiority to ADR in the standard murine L1210 and P388 tumor model systems (5). The high percentage of long-term survivors ("cures") produced by AD 32 in the L1210 system (5) continues to be observed in these laboratories, and our findings have now been confirmed in at least two independent studies (ref 6, and personal communication from F. M. Schabel and W. R. Laster, Southern Research Institute, Birmingham, Ala). Toxicity studies in rabbits have shown that AD 32 causes less toxicity in general and much less cardiomyopa-

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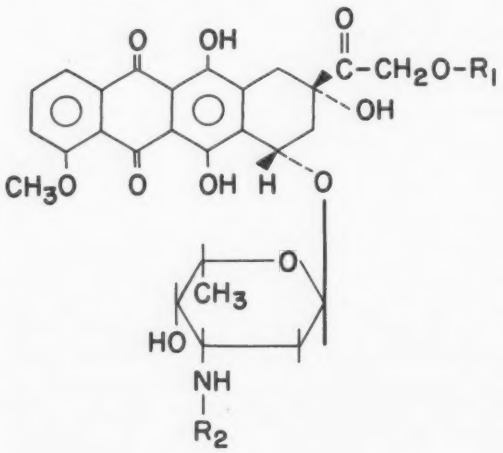
thy than similar or smaller cumulative doses of ADR when animals are given multiple doses of the two agents at equimyelotoxic dose levels (I. C. Henderson, M. Billingham, M. Israel, et al, unpublished results). Based upon its effectiveness in murine tumor systems and its reduced cardiac toxicity compared to ADR, AD 32 has been approved for clinical investigation, and phase I studies have begun at the Sidney Farber Cancer Institute using the Emulphor formulation described in this report.

Although similar to ADR in molecular structure (fig 1), AD 32 shows a number of significant biologic differences. AD 32, for example, essentially does not bind to isolated double-helical DNA (7), whereas for ADR, binding is presumed to be its mechanism of action (8). AD 32 rapidly enters live cultured cells and localizes exclusively in the cytoplasm, in contrast to the slow uptake of ADR and its localization in the nucleus (9). Recent pharmacokinetic studies (10,11) have established that ADR is not a significant metabolite of AD 32 in rodents.

The purpose of the present report is to provide additional evidence for the therapeutic superiority of AD 32 over ADR in experimental murine tumor systems, to demonstrate that this differential effect, at least in the L1210 system, is not dependent upon drug formulation, and to present some comparative mouse toxicity data.



Adriamycin: $R_1 = H$
$R_2 = H$

AD 32: $R_1 = COCH_2CH_2CH_2CH_3$
$R_2 = COCF_3$

FIGURE 1.—Structural relationship of ADR and AD 32.

MATERIALS AND METHODS

Animals

Male mice of the following strains were obtained from the Jackson Laboratory, Bar Harbor, Me: DBA/2, C57BL/6 × DBA/2 F_1 hybrids (BDF_1), AKR, and AKR × DBA/2 F_1 ($AKD2F_1$). Mice were housed no more than ten per cage in a controlled environment and allowed free access to water and laboratory chow. Animals used in these experiments had an average weight of 20 g.

Tumor Cell Lines

The L1210 cell line was obtained regularly from Arthur D. Little, Inc, Cambridge, Mass, courtesy of Mr. I. Wodinsky. Tumor was passaged weekly in DBA/2 mice and replaced periodically, as per established protocols (12). BDF_1 mice bearing an ADR-resistant P388 subline (P388/ADR) were obtained from Southern Research Institute, Birmingham, Ala, courtesy of Dr. F. M. Schabel, and the tumor line was passaged weekly in mice of the same strain. The Ridgway osteogenic sarcoma is maintained at the Sidney Farber Cancer Institute by serial transplantation of tumor fragments in AKR mice, as previously described (13).

Drugs

ADR and actinomycin D were supplied as clinically formulated drugs by the Division of Cancer Treatment, NCI, and were dissolved and used in 0.9% sodium chloride solution unless otherwise indicated; with ADR, no difference in biologic effect has been observed here between bulk material and formulated drug, which also contains lactose. The AD 32 used was prepared in these laboratories according to previously described procedures (5,14) or was provided to us by Farmitalia, Milan, Italy; Farmitalia-prepared samples were equivalent in all chemical, physical, and chromatographic properties with material prepared in our own laboratory. AD 32 samples of ≥ 98% purity (by high-performance liquid chromatographic assay) were used throughout. AD 32 was formulated in 10% aqueous Tween 80 or Emulphor-ethanol-saline as indicated. Emulphor-ethanol, as a 1:1 concentrate, was provided by Mr. J. Paul Davignon, Division of Cancer Treatment, NCI. When this concentrate is diluted with 9 parts of saline, the resulting mixture can be used

for formulations of AD 32 containing up to a working limit of 3.5 mg/ml; dilution of the concentrate 1:4 with saline allows up to 7.0 mg of AD 32 to be dissolved per milliliter of vehicle.

Normal Animal Toxicity

In three separate experiments, groups of BDF_1 mice were given either AD 32 in 10% aqueous Tween 80 or ADR in 0.9% sodium chloride as single or multiple ip injections; the animals were observed until death or were killed after 1 year. In two experiments, additional animals were included, so that, survival permitting, two animals at each dose level could be killed on Days 7, 14, 21, and 42. All animals were autopsied and evaluated for gross pathology.

In Vivo Antitumor Assays

Comparative antitumor activity for AD 32 and ADR against the L1210 and P388/ADR lymphocytic leukemias was determined by measuring the median percent increase in lifespan (ILS) of BDF_1 mice inoculated ip with 1×10^5 L1210 or 1×10^6 P388/ADR leukemic cells relative to untreated controls. The Ridgway osteogenic sarcoma was implanted by trocar into the thigh of $AKD2F_1$ mice. Agents were administered ip throughout. The assay procedures for the ip implanted leukemias were identical to standard NCI protocols (ref 12, pp 7–9) for L1210 and P388 tumor systems with treatment commencing on the first day after tumor implantation and continuing once daily for 4 consecutive days. For Ridgway osteogenic sarcoma, treatment was delayed until all animals had palpable tumor with a median diameter of 10 mm; for this tumor the number of complete responses and their duration were measured, in addition to prolongation of survival.

Percent ILS was calculated according to the equation % ILS = $100 \times (T/C - 1)$, where T equals the median survival time in days of the treatment group and C equals the median survival time in days of the 0-dose control group.

Effects of AD 32 and ADR on In Vivo DNA Synthesis

BDF_1 mice were inoculated with 1×10^5 L1210 leukemia cells and a single ip administration of drug was given 4 days later. The methodology for measuring ^{3}H-thymidine incorporation into normal and neoplastic tissues was a modification of that described by Chabner and Young (15). At intervals following treatment, four animals from each group received 50 microcuries of ^{3}H-thymidine (specific activity, 6 curies/mmol) ip. One hour after receiving the labeled nucleoside, mice were killed by cervical dislocation, and gastrointestinal mucosa, femoral bone marrow, and ascitic tumor cells were harvested. DNA was extracted from acid-insoluble material by the method of Schneider (16), and the amount present was determined colorimetrically by the method of Burton (17). A 0.5-ml portion of the extract was added to 10 ml of liquid scintillation cocktail (Aquasol, New England Nuclear Corp, Boston, Mass) and the tritium content was determined. Results were corrected by the addition of ^{3}H-toluene as an internal standard and expressed as the mean dpm/μg of DNA. Baseline values for ^{3}H-thymidine incorporation into intestinal mucosa, bone marrow, and ascitic tumor cells 4 days after implantation were (mean ± SE) 623 ± 70, 426 ± 50, and 17,000 ± 2000 dpm/μg respectively.

RESULTS AND DISCUSSION

Toxicity Studies

The comparative lethality of ip administered ADR and AD 32 in normal BDF_1 mice is shown in table 1. No toxic deaths were seen within the initial 60-day observation period with four daily doses of 20–90 mg/kg of AD 32; this dose range includes the therapeutic dose levels of AD 32 in L1210 and P388 tumor therapy studies. A dose of 120 mg/kg of AD 32 for 4 days produced 100% acute lethal toxicity (median survival, 7 days). Single doses of up to 300 mg/kg of AD 32 did not result in the death of any animal; scarcity of compound prevented further single-dose escalation.

In contrast, when ADR was given once daily for 4 days, median survival times of 21, 16, and 7 days were found for total ADR doses of 16, 24, and 32 mg/kg respectively. A single ip dose of ADR of 20 mg/kg was a lethal dose for 100% of the animals (median survival, 9 days).

Acute ADR toxicity was marked by the generally wasted appearance of the animal, and, on postmortem examination, by atrophy of the spleen and thymus and by dilatation of the bowel and bloody intestinal content suggestive of severe damage to the gastrointestinal mucosa. These effects were seen in all ADR-treated animals that died within the first 2 weeks following drug administration, as well as in animals killed on Day 7 after receiving 8.0 mg/kg × 4 and 20 mg/kg × 1. ADR-treated animals dying later than 2 weeks after drug administration appeared similarly wasted; they occasionally showed a small amount of opalescent fluid in the thoracic

and/or peritoneal cavity, but otherwise exhibited no gross abnormalities. In contrast to the findings with ADR, necropsy of AD 32-treated animals was unremarkable, except for wasting, atrophy of lymphoid organs, and hemorrhagic bowel content of animals at the 120 mg/kg × 4 dose level.

In one experiment, when median weight changes for normal BDF_1 mice treated with AD 32 were determined for 60 days after drug administration, a dose-related acute weight loss relative to controls was seen. The largest acute fall in weight (12%) occurred at the highest dose level used (80 mg/kg × 4). Animals in this group regained weight over the first 20–25 days, but three of ten animals died between Days 60 and 130. In another experiment, however, there were neither early nor late deaths among a group of animals receiving the higher dose of 90 mg/kg for 4 days. It should also be noted that, from L1210 tumor therapy trials, the long-term survivors documented in our earlier report (5) were eventually killed, some > 525 days after tumor inoculation and subsequent treatment, and were found on autopsy to be tumor-free and otherwise normal in all respects. These findings help to support our earlier contention (5) of an absence of AD 32-associated delayed toxicity in the mouse.

Because of inadequate supplies of drug a precise LD10 or LD50 for single-dose AD 32 was not determined. No deaths were seen in normal mice with AD 32 even at 15 times the 100% lethal single dose of ADR. In L1210 leukemia-bearing mice, an empirical AD 32/ADR toxicity ratio of about 12 was derived, based upon data pooled from 16 experiments (table 2). Toxic death in a tumor-bearing animal is defined as death occurring 15% or more prior to the median day of death of tumor-bearing untreated controls, as per established protocols (ref 12, pp 7–9).

Detailed comparative toxicity studies, including histology, with ADR and AD 32 have been carried out in rabbits, in connection with cardiotoxicity evaluation, and will be described in a future report.

TABLE 1.—Toxicity of AD 32 and ADR in normal BDF_1 mice (drugs administered ip, AD 32 in 10% aqueous Tween 80 and ADR in 0.9% sodium chloride; pooled data from 3 experiments)

Dose	No. of animals	No. of deaths through Day—			
		15	30	60	150
		AD 32			
mg/kg qd Days 1–4					
20	10	0	0	0	0
40	10	0	0	0	1
50	10	0	0	0	0
60	15	0	0	0	0
80	10	0	0	0	3
90	5	0	0	0	0
120	5	5*	—	—	—
mg/kg × 1					
90	10	0	0	0	0
145	10	0	0	0	0
150	5	0	0	0	0
300	5	0	0	0	0
		ADR			
mg/kg qd Days 1–4					
2.0	10	0	0	0	0
4.0	10	0	9	10	—
6.0	11	4	11†	—	—
8.0	14	14‡	—	—	—
mg/kg × 1					
5.0	10	0	0	0	0
10.0	10	0	0	0	0
15.0	10	2	2	4	4§
20.0	13	13¶	—	—	—

*All animals dead by Day 8.
†All animals dead by Day 18.
‡All animals dead by Day 10.
§4 of 6 150-day survivors died before the end of 1 yr.
¶All animals dead by Day 11.

TABLE 2.—Toxicity of AD 32 compared to ADR in L1210 leukemia-bearing mice (pooled data from 16 experiments)

Compound	No. of animals	Toxic deaths* (%)
AD 32 (mg/kg qd Days 1–4)		
20.0	5	0
30.0	12	0
40.0	24	0
50.0	25	4
60.0	23	9
70.0	19	47
80.0	7	86
ADR (mg/kg qd Days 1–4)		
0.5	5	0
1.0	12	0
2.0	72	0
2.5	37	5
3.0	95	3
4.0	118	5
5.0	56	29
6.0	38	68

*Toxic deaths are defined as those occurring 15% or more prior to the median day of death of untreated controls, as per standard NCI protocols (ref 12, pp 7–9).

Leukemia L1210 Studies: Effect of Vehicle

In contrast to ADR, AD 32 is water-insoluble as a result of the trifluoroacetyl substituent (which removes the basicity of the glycosidic amine) and the lipophilic 5-carbon ester side chain. Thus, an appropriate nontoxic surfactant medium (10% Tween 80) was used for solubilizing AD 32 for initial in vivo biologic evaluation (5). In view of the report by Riehm and Biedler (18) on the potentiation of drug effect by Tween 80, we examined whether the therapeutic superiority of AD 32 over ADR may be due to a vehicle effect. These studies are summarized in table 3. Experiments 1 and 2 compared four daily administrations of ADR at various doses, formulated in either saline or 10% aqueous Tween 80. No difference in biologic effect was seen between ADR in saline versus Tween 80 in either experiment.

Because of the hemolysis it is known to produce, Tween 80 is not an acceptable vehicle for iv use in man. With the cooperation of the Division of Cancer Treatment, NCI, a formulation for AD 32 involving a polyoxyethylated castor oil derivative (Emulphor), with ethyl alcohol and saline, has been developed. Comparison of ADR in saline and an Emulphor-alcohol formulation (experiment 3) similarly showed no difference. Experiment 4 showed that AD 32 in the Emulphor-alcohol formulation was therapeutically superior to ADR, as was AD 32 in the Tween 80 formulation.

Clinical trials in progress with AD 32 use a formulation consisting of 5% Emulphor-5% ethanol-90% saline, as was used with ADR in experiment 3. However, in experiment 4, AD 32 was formulated in 10% Emulphor-10% ethanol-80% saline in order to limit the volume of each injection. Parallel vehicle control studies with normal animals indicated that 0.1 ml of the 10% Emulphor formulation/10 g of body weight for 4 successive days, or 0.3 ml of the 5% formulation/10 g of body weight for 9 consecutive days, was nontoxic. The 5% Emulphor formulation alone (0.1 ml/10 g of body weight) was inactive in the L1210 system (experiment 3). A 10% Emulphor vehicle control group was not included at the time in experiment 4. However, from other studies in these laboratories, it is clear that 10% Emulphor, 0.1 ml/10 g of body weight for 4 days, has no effect on the survival of L1210 leukemia-bearing mice.

Our results with ADR, for which surfactant formulations do not show enhanced in vivo antitumor activity relative to the standard clinical saline formulation, are supported by the findings of Vecchi et al (6), but at variance with Pratesi et al (19), who claim to observe a vehicle effect.

It should be noted that, in the L1210 system, the optimal dose of ADR on the daily × 4 schedule can be somewhat variable (eg, as in table 3). In our experience, most often the optimal ADR dose on this schedule is 4.0 mg/kg daily. As can be seen in table 2, only 5% of tumor-bearing animals treated with ADR at 4 mg/kg × 4 appear to have toxic deaths, as defined in standard protocols (ref 12, pp 7–9). However, as described above and in table 1, this dose level is lethal to normal animals of the same strain as used in therapy trials. Based upon these convergent findings, it becomes obvious that the "optimal" dose of the ADR in tumor therapy trials, at least against L1210 leukemia, represents a major compromise between its antitumor efficacy and its latent toxicity. This apparently is not so for AD 32.

P388/ADR-Resistant Leukemia

AD 32 is therapeutically superior to ADR in the standard (sensitive) P388 in vivo assay (5). When evaluated against a P388 subline selected for ADR resistance, AD 32 produced an ILS of 118% and 130% in the optimal dose range of 40–50 mg/kg/day for 4 days in two of two experiments (table 4) and was superior to ADR at the dosages employed.

While 50–60 mg/kg for 4 days is the optimal (highest nontoxic) dose range for AD 32 in BDF_1 mice in the L1210 leukemia system, in the normally sensitive P388 leukemia system, and in the P388/ADR system employed here, AD 32 is often toxic

TABLE 3.—Effect of vehicle on the antileukemic activity in L1210 leukemia of ADR and AD 32 (BDF_1 male mice, 10^5 tumor cell inoculum)

Experiment No.	Drug (schedule)	Vehicle*	Each dose (mg/kg)	Mice/group	Median survival time in days (range)	% ILS	Survivors/total No. of mice	
							30-day	60-day
1	None (controls)	—	—	15	9.0 (8–10)	—	0/15	—
	ADR (qd Days 1–4)	Saline	2.0	5	13.0 (12–15)	44	0/5	—
			2.5	5	14.0 (13–17)	56	1/5	1/5
			3.0	5	14.0 (13–15)	56	0/5	—
			4.0	5	14.0 (14–15)	56	1/5	1/5
			5.0	5	15.0 (7–38)†	67	1/5	0/5
	ADR (qd Days 1–4)	Tween 80	2.0	5	13.0 (12–13)	44	0/5	—
			2.5	5	13.0 (12–15)	44	0/5	—
			3.0	5	14.0 (13–18)	56	0/5	—
			4.0	5	15.0 (15–27)	67	0/5	—
			5.0	5	16.0 (13–37)	78	1/5	0/5
2	None (controls)	—	—	15	10.0 (9–12)	—	0/15	—
	ADR (qd Days 1–4)	Saline	2.5	7	12.0 (8–15)	20	0/7	—
			3.0	7	13.0 (10–14)‡	30	0/7	—
			3.5	7	13.0 (11–14)	30	0/7	—
			4.0	7	14.0 (10–19)	40	0/7	—
			4.5	7	14.0 (11–16)	30	0/7	—
			5.0	7	13.0 (11–16)	30	0/7	—
	ADR (qd Days 1–4)	Tween 80	2.5	7	14.0 (12–17)	40	1/7	1/7
			3.0	7	15.0 (9–16)	50	0/7	—
			3.5	7	17.0 (12–20)	70	0/7	—
			4.0	7	12.0 (10–15)	20	0/7	—
			4.5	7	15.0 (11–21)	50	0/7	—
			5.0	7	13.0 (9–19)	30	0/7	—
3	None (controls)	—	—	15	9.0 (8–13)	—	0/15	—
	ADR (qd Days 1–9)	Saline	1.0	7	15.0 (10–18)	66	0/7	—
			1.5	7	18.0 (10–19)	100	2/7	2/7
			2.0	7	17.0 (13–27)	88	0/7	—
			2.6	7	14.0 (13–17)‡	55	0/7	—
			3.5	7	13.0 (10–16)	44	0/7	—
	ADR (qd Days 1–9)	5% Emulphor	0.0	7	9.0 (6–10)	0	0/7	—
			1.0	7	13.0 (12–21)	44	0/7	—
			1.5	7	17.0 (13–35)	88	1/7	0/7
			2.0	7	14.0 (11–18)	55	0/7	—
			2.6	7	14.0 (10–16)	55	0/7	—
			3.5	7	10.0 (5–11)	11	0/7	—
4	None (controls)	—	—	15	10.0 (9–15)	—	0/15	—
	ADR (qd Days 1–4)	Saline	2.5	7	18.0 (12–21)	80	0/7	—
			3.0	7	16.0 (12–21)	60	0/7	—
			3.5	7	14.0 (11–20)	40	0/7	—
			4.0	7	11.0 (8–11)	10	2/7	2/7
	AD 32 (qd Days 1–4)	10% Emulphor	30.0	7	— (18–28)	—	4/7	4/7
			40.0	7	36.0 (14–36)	260	3/7	3/7
			50.0	7	— (9–61)	—	5/7	5/7
			60.0	7	— (76,77)‡	—	6/6‡	6/6

*Volume injected = 0.1 ml/10 g of body weight; saline = 0.9% sodium chloride for injection; Tween 80 = 10% aqueous Tween 80; 5% Emulphor = Emulphor-ethanol-saline, 5:5:90 by volume; 10% Emulphor = Emulphor-ethanol-saline, 10:10:80 by volume.

†2 of 5 toxic deaths observed.

‡Does not include 1 injection death.

above 40 mg/kg when given daily for 4 days to similar hybrid animals. A possible explanation for the increased toxicity of AD 32 in P388 systems may relate to the greater tumor inoculum used in these assays (tumor cell inoculum of 10^6 cells for P388 and P388/ADR versus 10^5 cells for L1210).

Ridgway Osteogenic Sarcoma

The results of treatment of established Ridgway osteogenic sarcoma are detailed in table 5 and displayed in figure 2. Actinomycin D was used as a positive control in one experiment and resulted in a 100% complete response rate (loss of palpability), with a median duration of response of 29 days. All animals achieving a response ultimately relapsed and died of tumor with a median survival of 86 days (ILS = 136%), compared to a median survival for controls of 36.5 days. Two dose levels of AD 32 were compared with the optimal dose of ADR which would produce no late toxicity. Both AD 32 doses produced a 100% complete response rate of 12 days' duration with median survivals of 66 and 71 days, somewhat better than the 71% complete response

TABLE 4.—Effect of AD 32 and ADR on P388/ADR-resistant leukemia (BDF_1 male mice; 10^6 tumor cell inoculum)*

Experiment No.	Compound	Dose (mg/kg qd Days 1–4 ip)	Mice/group	Median survival time in days (range)	% ILS
1	None (controls)	—	10	11.0 (10–13)	—
	ADR	2.5	6	17.5 (13–21)	59
		3.0	6	17.0 (15–20)	54
		4.0†	6	16.5 (15–24)	50
		5.0†	6	14.5 (12–18)	32
	AD 32	30	6	25.0 (17–30)	127
		40	6	23.5 (17–38)	114
		50	6	24.0 (22–35)	118
		60	6	20.0 (8–23)	82
2	None (controls)	—	10	10.0 (10–11)	—
	ADR	2.5	7	15.0 (14–16)	50
		3.0	7	14.0 (13–19)	40
		3.5	7	15.0 (11–17)	50
		4.0	7	15.0 (13–16)	50
	AD 32	40	7	23.0 (21–30)	130
		50†	7	11.0 (7–22)	10
		60†	7	7.0 (2–7)	–30

*Tumor obtained through the courtesy of Dr. F. M. Schabel, Southern Research Institute, Birmingham, Ala.
†Toxic (> 15% median weight loss between Days 1 and 7).

TABLE 5.—Comparative therapy of established Ridgway osteogenic sarcoma* with ADR and AD 32

Compound	Dose (mg/kg)	Schedule	No. of mice	Complete responses† %	Complete responses† Median duration (days)	Survival (days) Day of death	Survival (days) Median	% ILS
None (saline control)	—	qd Days 16–19	10	—	—	28, 19, 36(3)§, 37, 39(2), 41(2)	36.5	—
ADR (saline)	3	qd Days 16–19	7	71‡	7‡	41, 49, 50, 51(2), 52, 62	51.0	+40
AD 32 (10% Tween 80)	50	qd Days 16–19	7	100‡	12‡	57(2), 62, 71(2), 75, 77	71.0	+95
	60	qd Days 16–19	7	100	12	58, 65(2), 66, 68(2), 77	66.0	+81
Actinomycin D (saline)	0.6	Day 16 only	7	100	29	76(2), 80, 86, 95, 98, 111	86.0	+136

*$AKD2F_1$ male mice implanted with 0.5-ml tumor suspension im; test agent administered ip.
†Defined as the absence of palpable tumor.
‡*See* fig 3 for a graphic representation of comparative induction and duration of response.
§Nos. in parentheses = No. of animals.

rate of 7 days' duration and 51-day median survival for ADR. In an independent study at the Southern Research Institute, AD 32 was found to be active in the Ridgway system, with optimal doses of AD 32 and ADR producing essentially the same response (F. M. Schabel and W. R. Laster, personal communication). In view of the clinical importance of ADR in the treatment of solid tumors, the activity of AD 32 in the Ridgway system is important, for it establishes the ability of AD 32 to act on solid tumors at sites distant from the point of administration.

Comparative Effects on DNA Synthesis in L1210-Bearing Mice

AD 32 and ADR were compared at a 10:1 dose ratio for their effects on ^{3}H-thymidine incorporation in rapidly proliferating neoplastic and normal tissues in L1210-bearing mice. As seen in figure 3, the two drugs produced both qualitatively and quantitatively similar effects on incorporation of the DNA precursor into gastrointestinal mucosa, bone marrow, and ascitic tumor cells. ADR is presumed to exert its antitumor effect through intercalation with DNA and subsequent inhibition of nucleic acid

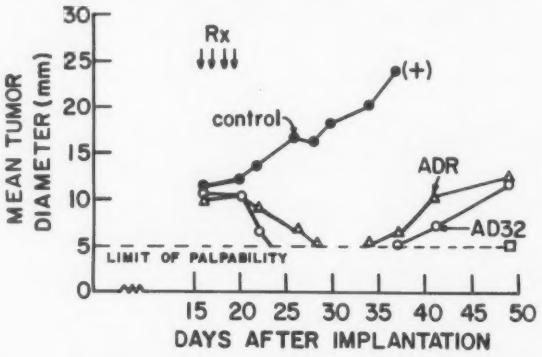


FIGURE 2.—Response of Ridgway osteogenic sarcoma (induction and duration of complete response) in AKD2F$_1$ mice. Each dose of ADR was 3 mg/kg; each dose of AD 32 was 50 mg/kg.

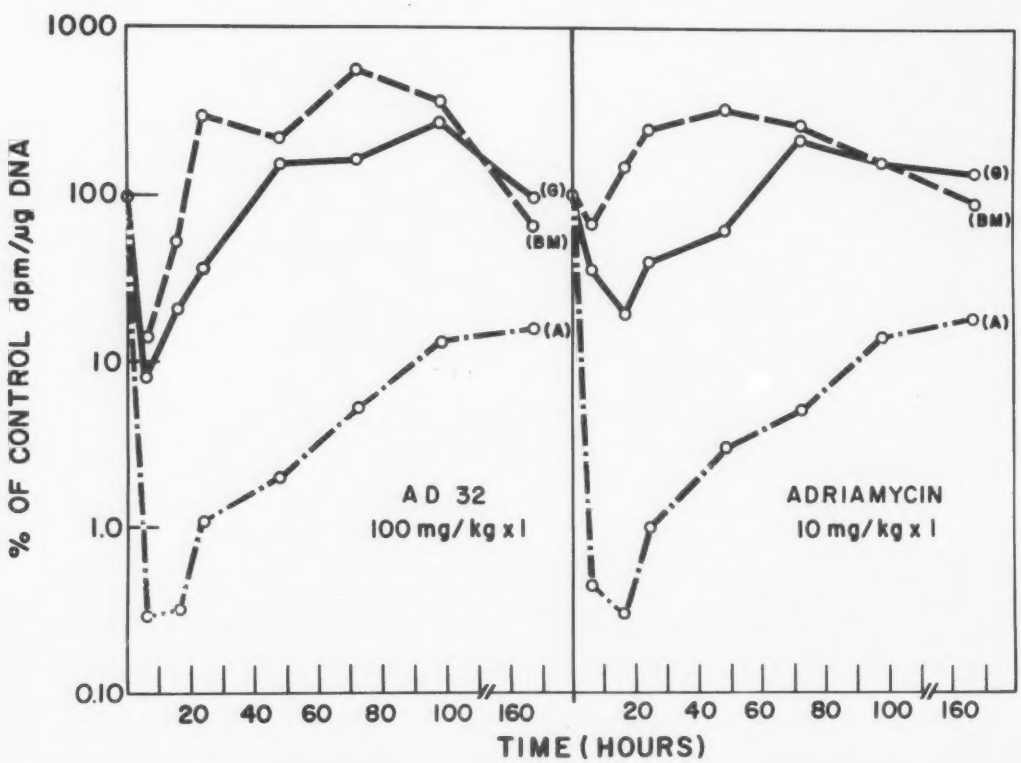


FIGURE 3.—Comparative effect of ADR and AD 32 on ^{3}H-thymidine incorporation in BDF$_1$ male mice bearing the L1210 leukemia. (A) = ascitic tumor cells; (BM) = femoral bone marrow; (G) = gastrointestinal mucosa.

synthesis. That AD 32 exerts an almost identical effect on DNA synthesis in vivo under defined experimental conditions is intriguing in light of evidence which shows that AD 32 binds poorly to DNA, if at all (7), and that its pharmacology differs significantly from that of ADR, with no evidence that ADR is a significant metabolite of AD 32 (10,11).

CONCLUSION

In addition to its activity in the standard murine P388 leukemia system, AD 32 is more effective than ADR against a P388 subline selected for ADR resistance. It is also active against the Ridgway osteogenic sarcoma, a solid tumor model. The therapeutic superiority of AD 32 over ADR in the murine L1210 system, previously reported by us and now confirmed by others, is not the result of differences in the formulation vehicles originally employed. In our hands, the effect of ADR on in vivo L1210 leukemia was not enhanced by formulation in surfactant-containing vehicle relative to saline, whereas AD 32 was as effective when formulated in an Emulphor-ethanol-saline vehicle as it was in 10% aqueous Tween 80.

Preliminary studies (20) also suggest that AD 32 may be more effective than ADR in the L1210 system under more demanding conditions, ie, when tumor is implanted iv or sc, or when treatment is delayed until Day 4 with ip implanted tumor. Our results now gain support from the work of Vecchi et al (6), who report similar findings of improved effectiveness of AD 32 over ADR against iv implanted L1210 and ip implanted tumor with delayed therapy when examined under somewhat different experimental conditions than had been used here.

The similarity of effect of AD 32 and ADR on DNA synthesis, as measured by ^{3}H-thymidine incorporation in normal and tumorous tissue, together with other data on comparative DNA-binding and pharmacology, suggests that the mechanism of action of AD 32, and perhaps of ADR as well, may be due to factors other than direct DNA complex formation.

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Chemotherapy of Disseminated Germinal Tumors of the Testis—Comparison of Vinblastine and Bleomycin With Vincristine, Bleomycin, and Actinomycin D [1,2]

Brief Reports and Preliminary Communications

Stuart C. Spigel, Ronald L. Stephens, Charles D. Haas, Stephen E. Jones, Daniel Lehane, Thomas E. Moon, and Charles A. Coltman, Jr [3,*]

On the basis of early reports (1)[4] concerning the efficacy of vinblastine and bleomycin (VB) used singly and in combination in the therapy for advanced testicular tumors, a pilot study utilizing these agents was undertaken at Wilford Hall US Air Force Medical Center (2). Eleven patients with nonseminomatous, germinal testis tumors were treated, and there were five complete and four partial responses. These pilot data stimulated the SWOG to embark on a randomized, prospective study of testicular carcinoma utilizing VB compared to actinomycin D, vincristine, and bleomycin (AVB). The AVB limb was selected to ascertain whether the nonmyelosuppressive drug, vincristine, could substitute for the myelosuppressive drug, vinblastine, allowing the addition of actinomycin D.

MATERIALS AND METHODS

All patients with measurable stage III (supradiaphragmatic or abdominal) metastatic germinal testicular carcinoma were eligible, provided they had had no prior exposure to any of the protocol agents. All but ten patients had dominant pulmonary metastases.

Chemotherapy was administered as follows.

VB limb.—vinblastine: 15 mg/m² divided into two daily iv bolus fractions and repeated every 21 days, eg, Days 1 and 2, and 22 and 23 (if there had been prior extensive radiotherapy, this drug was initiated at half dose); bleomycin: initial 5-unit test dose iv, then 15 units/m² iv twice weekly by 20-minute infusion until attainment of a 230-units/m² ceiling dose or 400 units total dose, then discontinue.

AVB limb.—actinomycin D: 0.35 mg/m² iv daily for 4 days repeated every 21 days (if there had been prior extensive radiotherapy, this drug was initiated at half dose); vincristine: 1.0 mg/m² iv weekly, timed to be given on the same day as but 6 hours prior to bleomycin; bleomycin: same as in VB limb.

Complete responders treated with the VB limb were carried to the ceiling dose of bleomycin and maintained with vinblastine alone indefinitely or until evidence of relapse. AVB limb complete responders were also carried to the bleomycin ceiling dose and maintained with actinomycin D. With 22 evaluable patients in the VB limb and 20 in the AVB limb, the VB regimen appeared significantly superior and the AVB limb was closed. Since that time, all additional patients have been enrolled in the VB limb.

RESULTS

A total of 70 patients were entered into the study between March 1973 and February 1975. One patient was considered not eligible. Of the eligible patients, 57 (83%) were fully evaluable and 11 (19%) were partially evaluable, giving a total of 68 patients included in this analysis. The reasons for partial evaluability in the 11 patients were early death (five), inadequate trial due to toxicity (one), lost to followup (three), and refused further therapy (two). One patient receiving the AVB limb was not evaluable because of inadequate data.

Table 1 gives the response rates for all patients by induction treatment and Dixon and Moore (3) classification. There is no statistical evidence of a difference between these complete response (CR)

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[3]This study was performed by the Southwest Oncology Group (SWOG). The following institutions participated in the study: US Air Force Medical Center-Keesler, Miss (*S. C. Spigel*); University of Kansas Medical Center, Kansas City (*R. L. Stephens* and *C. D. Haas*); University of Arizona Medical Center, Tucson (*S. E. Jones*); Baylor College of Medicine, Houston, Tex (*D. Lehane*); The University of Texas System Cancer Center, M. D. Anderson Hospital and Tumor Institute, Houston (*T. E. Moon*); and Wilford Hall US Air Force Medical Center, Lackland Air Force Base, Tex (*C. A. Coltman, Jr*).

[4]Agre K. Clinical summary of bleomycin. Bristol Laboratories, Syracuse, NY, Oct 1970.

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TABLE 1.—Response by treatment and cell type

	No. of responses (%)												
		VB limb*						AVB limb†					
			Cell type						Cell type				
Response	Total	All VB	I	II	III	IV	V	All AVB	I	II	III	IV	V
CR	27(40)	21(44)	2	13	3	2	1	6(30)	0	5	0	1	0
PR	16(24)	10(21)	0	5	1	3	1	6(30)	0	3	1	0	2
None	13(18)	8(16)	1	3	2	1	1	5(25)	1	0	3	1	0
Increasing disease	6(9)	3(6)	0	1	0	1	1	3(15)	0	3	0	0	0
Response unknown	6(9)	6(13)						0					
Totals	68	48	3	22	6	7	4	20	1	11	4	2	2

*6 patients receiving VB limb have no response listed: early death (5 patients) and inadequate trial due to toxicity (1 patient).
†1 patient receiving AVB limb has no response listed (not evaluable because of inadequate data).

rates or the CR plus partial response (PR) rates (all P values > 0.15). The median duration of CR was 48 weeks for the VB arm and 41 weeks for the AVB arm ($P = 0.76$). The median survival for VB patients was 63 weeks compared to 31 weeks for AVB patients ($P = 0.086$).

TOXICITY

Major toxic phenomena were myelosuppression, alopecia, nausea and emesis, neuropathy, and dermatitis. There were no drug-related deaths, though one patient in the VB limb was withdrawn from the study because of dyspnea which we felt was due to bleomycin toxicity following administration of a test dose. There were two additional instances of pulmonary toxicity attributable to bleomycin, both in AVB patients. Myelosuppression was not a significant problem among AVB recipients, but dermatitis occurred more commonly. Jaw or dental pain was limited to the VB limb and has previously been described in association with vinblastine (1).

DISCUSSION

The overall response rate was 35% (10% CRs) with chemotherapy for metastatic testicular carcinoma prior to the utilization of VB (2). The data reported here establish an overall response and CR rate of 65% and 44% in the VB limb, and 60% and 30% in the AVB limb, respectively. The hypothesis that the AVB limb might prove superior, because of the use of actinomycin D at full dose in addition to the nonmyelosuppressive drugs vincristine and bleomycin, was not proven. In view of the relative absence of myelosuppression and stomatitis in this arm, it is possible that the dose of actinomycin D selected was not optimal. However, using a higher dose of actinomycin D (0.4–0.6 mg/m^2/day × 5 days) in a similar AVB protocol, Blom and Brodovsky (4) have reported eight of 42 (19%) complete responders and 29 of 42 (69%) overall responders in this disease with a median survival time of 39 weeks, data remarkably similar to our own.

Samuels et al (5) have recently reported their experience with VB chemotherapy of 50 patients with stage III testicular carcinoma. The overall response rate was 75% with a 32% CR rate. The median survival was 48 weeks. Patients in CR for > 2 years have a high likelihood of cure, with 50% of the complete responders beyond 2 years having no evidence of disease. The median duration of CR in our study (VB, 48 weeks; AVB, 41 weeks) was similar to that reported by Samuels et al (5).

The VB regimen had a slightly higher CR rate (44% versus 30%) as well as a somewhat longer median survival (63 weeks versus 31 weeks) although these differences are not statistically significant. The addition of *cis*-dichlorodiammineplatinum(II) to VB has resulted in a 100% response rate (6).

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Oat Cell Carcinoma of the Lung: CNS Metastases in Spite of Prophylactic Brain Irradiation [1,2]

Brief Reports and Preliminary Communications

Martin Levitt,* Alec Meikle, Nevin Murray, and Brian Weinerman [3,4]

Recent trials (1–28) have demonstrated that oat cell carcinoma of lung (OCCL) is sensitive to many drugs and that more aggressive and toxic treatment produces higher remission rates and longer median survival times (MST). "Prophylactic" brain irradiation (XRT) although untried when this study began, seems now to be at least partly effective (18,21,26,29,30). Finally, it has been suggested that OCCL may be sensitive to "kinetically designed" treatment (31). This trial was begun in September 1974, based on the hope that it represented an "optimal multimodality strategy."

MATERIALS AND METHODS

The treatment group consisted of all 29 eligible patients who presented between September 1, 1974, and December 1, 1976. Eligibility requirements included no prior treatment; histopathologic confirmation of OCCL; needle marrow biopsy; liver, brain, and bone scans; and serum biochemistry determinations including BUN, alkaline phosphatase, SGOT, fasting blood sugar, and cortisol. Treatment was usually begun with XRT to the thoracic primary site and supraclavicular fossae. Ten equal doses of 400 rads were given from Days 1 to 5 and 22 to 26 with either a Linac 4-MV or a Betatron 32-MV source; "prophylactic" XRT (3000 rads) was delivered to the whole brain in ten doses from Days 1 to 12 using megavoltage delivered by either a cobalt 60 or a Linac 4-MV source. Then, allowing 2 weeks' "recovery," chemotherapy was begun using cyclophosphamide (CTX) (700 mg/m^2 every 3 weeks × two doses), CCNU (70 mg/m^2 orally, given with the first CTX dose only), and procarbazine (100 mg orally, Days 1–14, given to the first 17 patients). Three weeks after the second dose of CTX, adriamycin (60 mg/m^2) and vincristine (2 mg) were given iv. This schedule was given a second time 3 weeks later. Finally, after another 3-week interval, methotrexate (360 mg/m^2/24 hours with citrovorum factor rescue [32]) was given twice, once again with a 3-week interval. After 3 weeks the entire course was repeated. Treatment was then discontinued. Myelotoxic drug doses were reduced on a fixed scale for cytopenias as were adriamycin and vincristine for hepatic dysfunction. Significant neurotoxicity or nephrotoxicity did not occur. Complete remission (CR) was defined as the disappearance of all clinical and radiologic evidence of disease for ≥ 4 weeks. Partial remission (PR) was defined as a $\geq 50\%$ decrease in measurable disease for ≥ 4 weeks. The duration of remission was calculated from the time of *maximum* response to the first relapse; survival was calculated from the first date of treatment.

RESULTS

Of the 15 patients with limited disease (33), eight (53%) achieved CR and the remaining seven (47%) achieved PR. Subsequently, three of these patients relapsed in the CNS (all three PRs), eight in the thorax (three PRs and five CRs), two in the bone (both CRs), and three in the liver (two PRs and one CR). Of the thoracic recurrences, at least five were in sites previously irradiated. Of the 14 patients with extensive disease (26), one (7.1%) achieved CR and eight (57.1%) achieved PR; five (35.7%) did not respond (NR). Subsequently, two of these patients relapsed in the CNS (both PRs), six relapsed or progressed in the thorax (two NRs and four PRs), two relapsed in the bone (one NR and one PR), and four relapsed in the liver (two NRs and two PRs). Of the thoracic relapses and progressions at least three were in sites previously irradiated. The nonresponders survived from 5 to 30 weeks (median, 17). The median survival was 35 weeks for the limited-disease group and 23 weeks for the exten-

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[2]Previously presented in abstract form in the Proceedings of the American Society of Clinical Oncology, 1976 and 1977.

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sive-disease group, with one patient in the latter group still surviving and disease free at 126+ weeks. Two limited-disease patients died of drug toxicity and at autopsy they were found to have achieved CR. All other deaths were due to disease progression.

DISCUSSION

The survival of patients in this study is inferior to that of other recent studies (1–28) (median survivals in limited disease > 1 year in some studies). The failure of our high response rate to be accompanied by a significant improvement in survival may be due in part to two drug-related deaths in patients with CR. Further, "prophylactic" brain XRT appears to have been ineffective in this program in patients who did not achieve CR. In view of recent preliminary reports indicating effectiveness of prophylactic brain XRT (18,21,26,29,30), its apparent failure in this trial seems to be due to the gap between XRT and chemotherapy. Furthermore, at least eight of the 14 instances of recurrences or progression in the chest were in previously irradiated areas. In contrast, Holoye and Samuels (12) state "the importance of radiotherapy in the protocol seems very clear since only one of 16 patients with limited disease had recurrence within the chest in the previously irradiated area." In their study, chemotherapy preceded XRT. Thus, XRT given before chemotherapy does not appear to definitively influence relapse sites in either the brain or thorax. This observation may be relevant to failures of systemic adjuvant therapy in other neoplasms with high metastatic potential where primary treatment is regional in nature (eg, breast carcinoma).

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Antileukemia Therapy and Toxicology Studies in Mice With β-2′-Deoxythioguanosine [1,2,3]

Brief Reports and Preliminary Communications

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β-2′-Deoxythioguanosine (β-TGdR), an analog of deoxyguanosine, is an active agent against tumor and leukemic cells in animals (1).[5] β-TGdR is an S-phase specific agent (2) which apparently produces its lethal effects on mammalian cells as a result of its incorporation into DNA (3). β-TGdR has been shown to be an effective agent against acute leukemia in adults when administered as a rapid iv push daily for 5 days (4,5). Since cytosine arabinoside, also an S-phase specific agent, is an effective agent in man when administered by continuous iv infusion (6), we have investigated the antineoplastic activity of this route of administration of β-TGdR in mice with leukemia.

MATERIALS AND METHODS

$CD2F_1$ and BDF_1 male mice were obtained from Cumberland View Farms, Clinton, Tenn, and the Jackson Laboratory, Bar Harbor, Me, respectively. L1210 leukemic cells were transplanted by weekly ip injections of 10^5 cells in minimal essential medium (Grand Island Biological Co, NY) into BDF_1 mice. For chemotherapy studies, 5×10^4 L1210 leukemic cells in 0.1 ml of minimal essential medium were injected iv into the lateral tail veil of $CD2F_1$ mice. The β-TGdR solution was sterilized by filtration through a 0.22-μm Millex filter (Millipore Corp, Bedford, Mass). For the iv infusion experiments the β-TGdR solution was placed in 5-ml plastic syringes attached to polyethylene tubes with 25-gauge needles (Butterfly-25 Infusion Set, Abbott Laboratories, Chicago, Ill). The syringes were mounted onto Model 975 Compact Infusion Pumps (Harvard Apparatus Co, Millis, Mass). Mice were placed in a restraining cage, the 25-gauge needle was inserted into the tail vein, and the tail was immobilized by attaching a wooden tongue depressor with tape. There were ten mice per group in each experiment. The experiments were repeated two to three times and gave similar results.

RESULTS

The effect of a continuous iv infusion of β-TGdR for different durations on survival time of $CD2F_1$ mice with L1210 leukemia is shown in table 1. β-TGdR was infused at a constant rate of 110 μg/hour. The survival time of the leukemic mice increased with the duration of the β-TGdR infusion. β-TGdR infused at this rate for a duration of 1.0, 2.0, and 4.0 hours produced 31%, 52%, and 71% increases in lifespan (ILS) respectively of the leukemic mice. Injection of the $CD2F_1$ mice with only five L1210 leukemic cells was lethal to all the mice (R. L. Momparler and A. Gonzales, unpublished data) suggesting that only a few leukemic cells surviving chemotherapy could produce a fatal disease in these mice.

The effect of 6-hour iv infusions of β-TGdR at different dosages on the survival time of the leukemic mice is shown in table 2. The minimal effective dose of β-TGdR that produced a significant %ILS was about 4.9 mg/kg. This dose produced 27% ILS. A 6-hour iv infusion of β-TGdR at a dose of 47.4 and 453 mg/kg produced an 81% and 117% ILS respectively in the leukemic mice.

Table 3 shows the data on the toxicity of an 8-hour continuous iv infusion of β-TGdR at different doses in $CD2F_1$ mice. A dose of 35 mg/kg of β-TGdR was not lethal to the mice and did not produce any significant weight loss on Day 7 after therapy. Total doses of 680 and 1327 mg/kg of β-TGdR were the LD10 and LD90 respectively for the $CD2F_1$ mice. The group of mice treated at the LD90 dose of β-TGdR lost > 20% of their body weight on Day 5.

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[5]Rosenbaum C, and Carter SK. Clinical brochure: β-TGdR (NSC-71261). Prepared by the NCI, Bethesda, Md, 1969, pp 8–10.

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TABLE 1.—Effect of continuous iv infusion of β-TGdR for different durations on survival time of $CD2F_1$ mice with L1210 leukemia*

Duration of infusion (hrs)	β-TGdR dose (mg/kg)	Mean survival (days ± SD)	%ILS
—	0	7.7 ± 0.6	0
0.5	2.1	9.1 ± 1.0	18
1.0	4.2	10.7 ± 1.1	31
2.0	8.2	11.7 ± 0.4	52
4.0	16.3	13.2 ± 0.1	71
6.0	23.4	14.0 ± 0.4	82

*$CD2F_1$ male mice (10 wks old, 25–28 g) were injected iv with 5×10^4 L1210 leukemic cells on Day 0. Chemotherapy was started on Day 3. β-TGdR was dissolved in 0.45% NaCl at a concentration of 0.5 mg/ml, adjusted to pH 9.5, and infused at a flow rate of 0.22 ml/hr.

TABLE 2.—Effect of 6-hr iv infusion of β-TGdR at different doses on survival time of $CD2F_1$ mice with L1210 leukemia*

β-TGdR dose (mg/kg)	Mean survival time (days ± SD)	% ILS
0	7.2 ± 0.8	0
0.5	7.4 ± 0.9	3
4.9	9.1 ± 0.9	27
47.4	13.0 ± 0.8	81
453	15.6 ± 1.1	117

*$CD2F_1$ male mice (10 wks old, 26–29 g) were injected iv with 5×10^4 L1210 leukemic cells on Day 0. Chemotherapy was started on Day 3. β-TGdR was dissolved in 0.45% NaCl, adjusted to pH 9.5, and diluted to 0.01, 0.1, 1.0, and 10 mg/ml. β-TGdR was infused at a flow rate of 0.22 ml/hr.

DISCUSSION

When β-TGdR was infused at a rate of 4 mg/kg/hour the %ILS increased with the duration of the infusion (table 1). β-TGdR infused at this rate for 4.0 hours produced a 71% ILS. If this %ILS produced by β-TGdR represents a >90% (1.0 log) kill of L1210 leukemic cells the in vivo cytotoxic action of this analog would be classified as not self-limiting and would be in agreement with the report that β-TGdR does not block the progression of G_1 phase cells into S phase in vitro (2). This type of interpretation is based on the S-phase specificity of the cytotoxic action of β-TGdR (2) and the in vivo cell kinetics of L1210 leukemic cells. The average cell cycle time of L1210 leukemic cells is 11.8 hours with a $G_1 + M + G_2$ phase of 2.9 hours and a labeling index of 62% (7). An S-phase specific agent that is self-limiting would produce only a maximal 62% kill (fraction of cells in S phase) during a short infusion because the entry of the surviving $G_1 + M + G_2$ cells into S phase is blocked by the agent.

The minimal effective dose of β-TGdR that produced a significant %ILS in the leukemic mice when this agent was administered as a 6.0-hour infusion was 4.9 mg/kg (table 2). When pharmacokinetic data on β-TGdR in $CD2F_1$ mice become available it will be possible to use the data in table 2 to estimate the minimal plasma concentrations of β-TGdR that produce significant leukemic cell kill for a 6-hour iv infusion.

A 6.0-hour iv infusion of β-TGdR at a total dose of 453 mg/kg produced a 117% ILS which is a very potent antileukemic effect, suggesting that a continuous infusion is an effective way to administer this analog in leukemic mice. This dose of β-TGdR was not very toxic since it was considerably below the LD10 of 680 mg/kg administered as an 8.0-hour infusion to nonleukemic mice (table 3). We are currently using these preclinical data to assist in the evaluation of β-TGdR in a phase I study in pediatric patients with malignant disease.

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TABLE 3.—Toxicity of 8-hr continuous iv infusions of β-TGdR at different doses in $CD2F_1$ mice*

β-TGdR dose (mg/kg)	Mean survival time (days ± SD)	No. of survivors/ No. treated	LD level	Weight change, Day 5 (%)
185	> 35.0	10/10	LD0	+3.2
353	> 35.0	10/10	LD0	–2.2
680	5.2	9/10	LD10	–8.7
1327	3.8 ± 2.6	1/10	LD90	–22.9

*$CD2F_1$ male mice (9 wks old, 22–26 g) were administered an 8-hr iv infusion of β-TGdR. The β-TGdR was dissolved in 0.45% NaCl, adjusted to pH 9.5, and diluted to 2.5, 5.0, 10, and 20 mg/kg. β-TGdR was infused for 8 hrs at a flow rate of 0.22 ml/hr.

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Effect of ACNU on Primary Lung Cancer, Mesothelioma, and Metastatic Pulmonary Tumors [1,2,3]

Brief Reports and Preliminary Communications

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Recently, it has been revealed that nitrosourea derivatives such as BCNU, CCNU, and methyl-CCNU have antitumor activity in a variety of malignant tumors (1–4). ACNU, a water-soluble nitrosourea derivative developed in the central laboratory of Sankyo Co, Ltd, has been highly effective against L1210 leukemia (5), and according to the report by the ACNU phase I study group in Japan, the clinical maximal tolerable dose of ACNU was determined to be 3 mg/kg/dose (6). In the present study, ACNU was administered to patients with primary lung cancer, mesothelioma, or metastatic pulmonary tumor. Consequently, the antitumor and toxic effects of ACNU were evaluated clinically.

PATIENTS AND METHODS

Of the 56 patients entered in this study 32 patients had inoperable primary lung cancer, two patients had mesothelioma, and 22 patients had metastatic pulmonary tumor; they had been hospitalized in the National Cancer Center Hospital from October 1974 to February 1977 (table 1). The patients' ages ranged from 28 to 75 years, and there were 36 men and 20 women. Almost one half of the patients had already been treated with anticancer agents. ACNU was administered iv according to the following therapeutic schedules: (*a*) 0.4–1.0 mg/kg/day more than two times; (*b*) 2 mg/kg/week more than two times; (*c*) 3 mg/kg as a single injection. Response to treatment was determined as a decrease in diameter of the tumor objectively measurable on chest roentgenogram or by palpation. Complete response (CR) was defined as the disappearance of all disease. Partial response (PR) was defined as a 50%–99% decrease in the sum of the products of the length and width of measurable tumors. No response (NR) was defined as no change or $< 50\%$ reduction in measurable tumor. Wbc, platelet, and differential cell counts were checked before therapy and weekly after therapy. Urinalysis and blood chemistry were also checked before therapy and every 2 weeks after therapy.

RESULTS (tables 1 and 2)

The 56 patients reported here were all evaluable. In seven patients receiving daily divided doses of ACNU, no effective regression was observed. The response rate in 49 evaluable patients receiving ACNU at > 2 mg/kg/dose was 18.4% (nine of 49 patients) if response was defined as the patient having exhibited $> 50\%$ reduction in diameter of the tumor. All the responders with primary lung cancer were histologically classified as small cell carcinoma. An effect of ACNU on metastatic pulmonary tumor was observed in two patients with uterine carcinoma and in one patient each with laryngeal carcinoma, pharyngeal carcinoma, and fibrosarcoma. The onset of the effect was noted between 1 and 3 weeks after initiation of ACNU administration. The duration of the effect varied from patient to patient, and ranged from 2 to 34 weeks. No correlation was noted between the histologic tumor type and the duration of the effect. Leukocytopenia was noted in only one of seven patients in the daily divided dose group, whereas 60% of the 2-mg/kg/week group and 41.2% of the 3-mg/kg group had leukocytopenia. In most instances, leukocytopenia appeared about 4–5 weeks after ACNU administration and its nadir was noted after the fifth week following administration. Thrombocytopenia was noted in only two of seven patients in the daily divided dose group; however, it was

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[3] ACNU: NSC-245382; CAS reg. No. 55661-38-6; 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea, hydrochloride. Supplied by the Central Laboratory of Sankyo Co, Ltd, Tokyo, Japan.

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TABLE 1.—Effect of ACNU on primary bronchogenic carcinomas, mesothelioma, and metastatic pulmonary tumors

Tumor type	ACNU dosage											
	3 mg/kg				2 mg/kg/wk				0.4–1.0 mg/kg/day			
	No. of—				No. of—				No. of—			
	Patients	CR	PR	NR	Patients	CR	PR	NR	Patients	CR	PR	NR
Bronchogenic carcinoma												
Squamous cell carcinoma	5	0	0	5	2	0	0	2	3	0	0	3
Adenocarcinoma	6	0	0	6	6	0	0	6	1	0	0	1
Small cell carcinoma	6	1	1	4	3	2	0	1				
Mesothelioma	2	0	0	2								
Metastatic pulmonary tumor												
Uterine carcinoma	5	0	1	4	2	0	1	1				
Nasopharyngeal carcinoma	2	0	1	1								
Laryngeal carcinoma					1	0	1	0				
Renal carcinoma	1	0	0	1					1	0	0	1
Tongue carcinoma	1	0	0	1								
Parotid gland carcinoma	1	0	0	1								
Pancreas carcinoma	1	0	0	1								
Fibrosarcoma					1	1	0	0				
Chondrosarcoma	2	0	0	2								
Osteosarcoma	1	0	0	1					1	0	0	1
Liposarcoma									1	0	0	1
Leiomyosarcoma	1	0	0	1								
Total	34	1	3	30	15	3	2	10	7	0	0	7

TABLE 2.—ACNU toxicity

Toxicity	ACNU dosage		
	3 mg/kg (34 patients)	2 mg/kg/wk (15 patients)	0.4–1.0 mg/kg/day (7 patients)
Leukocytopenia			
No. of patients with leukocytes/mm³—			
2000–4000	10	8	1
< 2000	4	1	0
Total (%)	14(41.2)	9(60)	1(14.2)
Nadir*	5.2 ± 0.1	5.1 ± 0.4	
Thrombocytopenia			
No. of patients with thrombocytes × 10³/mm³—			
50–70	5	3	1
30–50	5	0	0
< 30	9	6	1
Total (%)	19(55.9)	9(60)	2(28.4)
Nadir*	4.1 ± 0.1	4.5 ± 0.5	

*Nadirs are expressed as wks (mean ± SE) from start of ACNU administration.

noted in 60% of the 2-mg/kg/week group and in 55.9% of the 3-mg/kg single administration group. The onset of thrombocytopenia appeared between the third and fourth week following ACNU administration with the nadir occurring after the fourth week following ACNU administration. The onset and nadir of the decrease in thrombocyte counts were noted 1 week earlier than those of the leukocyte counts. Almost no abnormal findings in blood chemistry due to ACNU administration were observed except for the slight increases of SGOT and SGPT noted in only one of 56 patients. Also, no renal dysfunction attributed to ACNU administration was detected. Patients temporarily complained of subjective toxic effects such as anorexia (13 patients), nausea (ten patients), vomiting (six patients), lassitude (four patients), and chest pain (one patient) which were attributed to ACNU administration.

DISCUSSION

ACNU produced a response rate of 18.4% (CR, 8.2%; PR, 10.2%) when it was administered in large intermittent doses in patients with advanced primary lung cancer or other malignancies. The response rate for patients with advanced lung cancer who received 2–3 mg/kg of ACNU intermittently was 14.3%; this was similar to that of other nitrosourea derivatives given by single administration (BCNU, 20%; CCNU, 13%; methyl-CCNU, 11%) (4). Histologically, all of the responders with advanced lung cancer had small cell carcinomas (response rate, 44.3%). ACNU was also effective for some kinds of metastatic pulmonary tumors such as uterine carcinoma, nasopharyngeal carcinoma, laryngeal carcinoma, and fibrosarcoma. The side effects of delayed myelosuppression and temporary gastric complaints were also similar to those of other nitrosourea derivatives. Our conclusion from this work is that the intermittent administration of ACNU (2–3 mg/kg) at intervals of 5–6 weeks may provide beneficial therapeutic results in induction therapy for small cell carcinoma of the lung and metastatic pulmonary tumors. Furthermore, it will be interesting to explore combination chemotherapy using ACNU for advanced malignancy.

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Adriamycin-Cyclophosphamide Treatment of Squamous Cell Carcinoma of the Cervix [1,2]

Brief Reports and Preliminary Communications

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The combination of adriamycin and cyclophosphamide has proven to be a successful therapy for advanced adenocarcinomas of the breast, ovary, and endometrium (1–4). There are no detailed reports of the adriamycin-cyclophosphamide combination in the treatment of recurrent squamous cell carcinoma of the cervix although the Gynecologic Oncology Group has been carrying out such a study (5). We have treated ten patients with far-advanced cervical cancer with this combination and have documented only two objective responses.

MATERIALS AND METHODS

Ten consecutive patients with biopsy-proven recurrent (ie, postradiation therapy of curative intent) and/or metastatic squamous cell carcinoma of the cervix were entered into this study at the Cancer Research Institute, University of California Hospitals (patients 1–7, table 1) and the Section of Hematology-Oncology, University Health Sciences Center (Ariz) (patients 8–10, table 1) between 1974 and 1976. No patient had received chemotherapy prior to adriamycin-cyclophosphamide. All patients had measurable disease on the basis of pelvic examination or chest roentgenogram.

All patients with no pelvic radiation therapy within 2 years of chemotherapy were given adriamycin, 40 mg/m^2 iv on Day 1, and cyclophosphamide, 200 mg/m^2 orally daily on Days 3–6, every 3–4 weeks as tolerated. Four patients with a history of recent radiation therapy received adriamycin at 20 mg/m^2 with a dose escalation at each new course (as tolerated) of 10 mg/m^2. Although the highest total dose of adriamycin was 320 mg/m^2 in patient 8 (table 1), the study protocol called for termination of adriamycin at a total dose of 500 mg/m^2.

RESULTS

Staging, chemotherapy, and response data for the ten study patients appear in table 1. There was only one partial responder (patient 4). She experienced a 75+% reduction in the size of her pelvic mass for 12 months. Unfortunately, she developed reversible congestive heart failure at a total dose of only 217 mg/m^2 of adriamycin, causing the discontinuation of this drug. One other patient had a 25% reduction in the size of her pulmonary metastases which lasted 3 months.

Only patient 1 (table 1) developed important leukopenia and thrombocytopenia. Despite a decrease in her adriamycin dose to 20 mg/m^2, the wbc count dropped to 1700/mm^3 within 1 week of her sixth course of chemotherapy and she died from both intracerebral and bladder hemorrhaging when her platelet count fell to 10,000/mm^3. No other patients experienced hemorrhagic cystitis.

DISCUSSION

Our 20% partial plus improved response rate in this study with adriamycin-cyclophosphamide is in the range of that achieved by either drug used alone (6,7). Wallace et al have made a preliminary report of a Gynecologic Oncology Group randomized study comparing adriamycin, adriamycin-vincristine, and adriamycin-cyclophosphamide in the treatment of advanced cervical cancer (5). There were no statistical differences in median progression-free interval or survival durations between the three treatment groups.

In view of the high response rates (ie, up to 60% partial remissions) achieved in advanced cervical cancer with mitomycin C-vincristine-bleomycin (8) and bleomycin-methotrexate (9), the poor results of our study suggest that the adriamycin-cyclophosphamide combination has no important role in the treatment of this disease.

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TABLE 1.—Data on staging, chemotherapy, and response in 10 cervical cancer patients

Patient No.	Age (yrs)	Disease stage at diagnosis	Measurable disease	Initial adriamycin dose (mg/m^2)	Escalation of dose	No. of courses	Response status
1	35	II	Lung, bone	40	No	6	Improved*
2	58	II	Pelvic mass	20	Yes	4	Progression
3	56	II	Pelvic mass	40	No	5	Progression
4	54	I	Vaginal vault	40	No	6	Partial†
5	35	I	Vaginal vault	40	No	2	Progression
6	63	I	Inguinal mass	20	Yes	3	Progression
7	57	IV	Lung, bone	20	Yes	4	Progression
8	58	I	Lung	40	No	8	Progression
9	53	III	Vagina	40	No	2	Progression
10	55	II	Pelvic mass	20	Yes	2	Progression

*Improved = 25%–50% decrease of tumor size associated with symptomatic improvement.
†Partial remission = ≥ 50% shrinkage of tumor for at least 2 mos.

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Intra-arterial Chemotherapy With Concomitant Use of Vasoconstrictors for Liver Cancer [1]

Brief Reports and Preliminary Communications

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Evidence has been presented that intrahepatic arterial administration of antitumor chemotherapeutic agents, especially cytocidal drugs, for the treatment of nonresectable tumors of the liver is a more effective and less hazardous procedure than systemic infusion (1). This is based upon the fact that liver tumors, either primary or metastatic, are mainly nourished by the hepatic artery (2). Although systemic side effects might be minimized with this procedure, it still causes cytotoxicity in normal liver parenchyma, which is one of the factors limiting the dose of chemotherapeutic agents.

On the other hand, newly formed blood vessels in tumor tissue are usually considered to be less responsive to vasoactive drugs than those in normal liver tissue. The administration of vasoconstrictors may induce a relative increase of blood flow in the tumor compared with the normal liver tissue. During this period, accordingly, chemotherapeutic agents may perfuse the tumor tissue much more than without vasoconstrictors. This study was performed to test this hypothesis.

MATERIALS AND METHODS

White rabbits of either sex weighing about 2.5 kg were used. Under iv anesthesia with pentobarbital sodium, 25 mg/kg, 0.5×10^6 cells of VX2 carcinoma (3) were implanted into a hepatic lobe. Three weeks after transplantation, the animals were divided into the following four groups: *Group I (five rabbits)*—serial hepatic arteriograms were taken after the injection of 20 μg of epinephrine into the hepatic artery (the most striking effect occurred within the first few minutes); *Group II (five rabbits)*—mitomycin C, 6 mg/kg, was injected into the hepatic artery immediately after administration of the same dose of epinephrine; *Group III (six rabbits)*—the same procedures without the injection of epinephrine were performed as a control of Group II; *Group IV (six rabbits)*—mitomycin C, 4 mg/kg, was injected into the hepatic artery in three animals with and in another three without 20 μg of epinephrine.

In Groups II and III, after rapid exsanguination, about 1 g of normal liver parenchyma and tumor tissue were taken. In Group IV, peripheral venous blood was withdrawn serially. The concentrations of mitomycin C in normal liver parenchyma, tumor tissue, and blood were determined by the bioassay method (4). The statistical comparison for significance was carried out according to the Mann-Whitney U test (5).

RESULTS

In Group I, serial hepatic arteriography demonstrated that the neovasculatures in the tumors are less responsive to epinephrine than the vessels of normal liver parenchyma, though the quantitative evaluation is rather difficult. The most prominent effect of the drug was confirmed to occur within several minutes after the injection; an increased opacification of tumors was observed during this period. Accordingly, mitomycin C was injected immediately after the infusion of epinephrine in Groups II and IV. The administration of epinephrine substantially reduced the concentration of mitomycin C in the normal liver parenchyma, but it did not increase the concentration of the drug in the tumor tissue. The tumor:liver ratio of the concentration of mitomycin C in each rabbit varied from 11.3 to 198.5 (mean 67.6) in Group II, and from 3.42 to 11.0 (mean 6.53) in Group III (table 1). Thus, the vasoconstrictor seemed to bring about a relative increase of the antitumor agent in the transplanted liver tumors. On the other hand, the use of epinephrine caused a twofold increase in the concentration of mitomycin C in the blood: 0.80 μg/ml without versus 2.20 μg/ml with epinephrine 5 minutes after mitomycin C, and 0.48 μg/ml without versus 0.84 μg/ml with epinephrine 10 minutes after mitomycin C.

DISCUSSION

The present investigation showed that the infusion of epinephrine into the hepatic artery alters the distribution of blood flow in the tumor-bearing

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TABLE 1.—Concentrations of mitomycin C in tumor and normal liver tissues and their tumor:liver ratio

Group	Concentration of drug (μg/g of tissue) in—		Ratio
	Liver	Tumor	
II	0.03	0.34	11.3
	0.04	7.97	198.5
	0.17	4.27	25.1
	0.03	1.51	50.3
	0.06	3.18	53.0
Mean	0.07*	3.45	67.6†
III	2.25	7.70	3.42
	1.21	7.58	6.26
	0.02	0.22	11.0
	0.25	0.89	3.56
	0.89	6.09	6.84
	0.48	3.89	8.10
Mean	0.85*	4.40	6.53†

*P = 0.041 (Mann-Whitney U test).
†P = 0.002 (Mann-Whitney U test).

liver, more in tumor and less in normal liver parenchyma, by constricting mainly the normal arteries. The preceding administration of epinephrine caused a decrease of mitomycin C concentration in the normal liver tissue but no change in the tumor tissue, thus indicating a relative increase in the tumor:liver ratio of mitomycin C concentration. That there was no absolute increase of the drug in the tumor tissue does not mean this procedure is useless since the total amount of the drug perfused in the tumor must be determined by integrating the concentration until the effective content of mitomycin C disappears from the blood. The increased concentration of mitomycin C in the blood with epinephrine probably indicates a decreased inactivation of the drug in the normal liver due to the decreased perfusion. Thus, the use of epinephrine seems to cause more selective perfusion of oncolytic agents. Although the safety of this maneuver must be further elucidated, it might be utilized in the general clinical trial.

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Levels of Two Plasma Fucosyltransferases as an Index of Disease Status in Acute Myelogenous Leukemias[1,2]

Brief Reports and Preliminary Communications

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The fucosyltransferases are enzymes which catalyze the transfer of the sugar fucose from GDP-fucose onto appropriate glycoprotein or carbohydrate acceptors. Elevated levels of plasma fucosyltransferase have been found in plasma of tumor-bearing animals and patients with neoplastic disease (1–4). The observed elevation may be related to shedding of membrane components and membrane-bound enzymes into plasma (5) since many glycosyltransferases are associated with cell-surface components (6–10), or a more specific secretory process may be involved. We have measured the levels of two fucosyltransferases in plasma from patients with acute myelogenous leukemia, using a procedure which corrects for endogenous enzyme acceptors. Both of the enzymes studied here were previously found in plasma from normal donors (11,12). Our data indicate a preferential elevation of the H gene-specified fucosyltransferase (12) in plasma of patients with untreated or nonresponding leukemias. This enzyme catalyzes transfer of fucose from a GDP-fucose donor onto terminal galactose residues (Fuc→Gal) of desialated fetuin and analogous acceptors. Another fucosyltransferase (9,11–13) which transfers fucose onto acceptors with terminal *N*-acetylglucosamine residues (Fuc→GlcNac) was elevated in plasma of patients during regeneration of normal marrow during drug-induced remissions.

MATERIALS AND METHODS

Plasma Samples

Plasma samples were obtained from normal donors and from patients in three clinical stages: untreated, nonresponding to drug therapy, and in remission. Patients in the first two classifications had marrow myeloblast levels ranging from 5% to 100%. In remission, the marrow contained < 5% myeloblasts or < 10% promyelocytes plus myeloblasts, with normal production of granulocyte, thrombocyte, and erythrocyte precursors.

Drug therapy consisted of iv administration of adriamycin plus vincristine on Day 1 and continuous iv infusion (7 days) of cytosine arabinoside and 5 days of oral prednisone. This regimen was repeated after 3-week drug-free intervals. Plasma samples from drug-treated patients were obtained just before the beginning of a new drug cycle. Untreated patients had had no prior drug therapy. Blood was anticoagulated with EDTA, erythrocytes, and platelets eliminated by successive centrifugations at 1500 and 10,000 × *g* for 10 minutes and the resulting plasma was stored at –70°C.

Enzyme Assays

A 150-μl volume contained 50 μl of plasma, 10 mM $MgCl_2$, 1 mM ATP, 10 mM EGTA, 1 μM GDP-[^{14}C]fucose (50,000 dpm), 50 mM cacodylate buffer, pH 7.0, and 0.5 mg of a fetuin-derived fucose acceptor. Incubations were carried out at 37°C for 60 minutes, sometimes in the presence of 10 mM *N*-ethylmaleimide (as described in ref 14). The product was separated from the radioactive precursor on columns of Dowex 1 (14).

RESULTS AND DISCUSSION

The data shown in figure 1 indicate an elevation in GDP-fucose:galactoside (Fuc→Gal) enzyme activity in plasma of patients with untreated or nonresponding acute myelogenous leukemias. Using another series of patients with nonresponding acute myelogenous leukemias (data not shown) with 90%–100% marrow blast cells, enzyme levels in the 1300–1800 range (counts/minute/50 μl of plasma) were found. In a more detailed report (4) we describe a positive correlation between the level of this enzyme versus the percent of marrow myeloblasts in individual patients. The enzyme level fell to control values during drug-induced remissions. In contrast,

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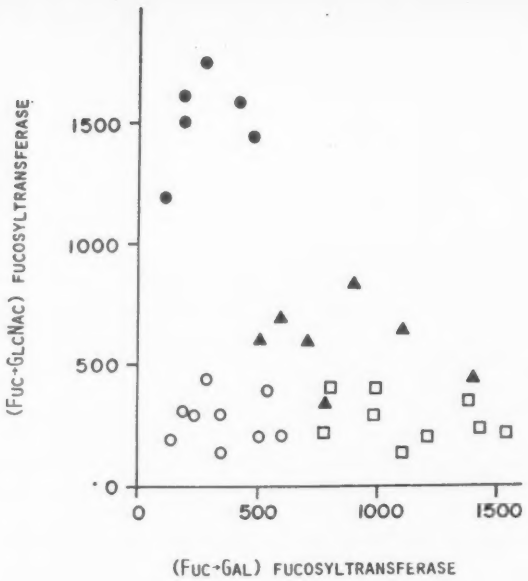

FIGURE 1.—Level of plasma GDP-fucose:galactoside (Fuc→Gal) and GDP-fucose:*N*-acetylglucosaminide (Fuc→GlcNac) fucosyltransferases in terms of [counts/min/50 μl of plasma] of radioactivity incorporated from GDP[^{14}C]fucose into glycoprotein acceptors during 60-min incubations at 37°C. Data are shown for controls (○) and for patients with untreated (□) and nonresponding (▲) acute myelogenous leukemia, and in drug-maintained remission (●). Using discriminant analysis, the significance of this classification was $P = 0.001$; $\chi^2(9) = 80$.

the plasma level of the GDP-fucose:*N*-acetylglucosaminide fucosyltransferase (Fuc→GlcNac) was not elevated during the acute phase of the disease, but was elevated during regeneration of a normal marrow population following a period of intensive drug therapy. Our patients in remission were usually maintained by periodic drug therapy. In one case, the level of this enzyme did fall to the control range during a 5-week drug-free interval.

Using a low molecular-weight fucose acceptor (15), Kuhns et al (16) reported that the plasma level of GDP-fucose:galactoside fucosyltransferase fell below the control range in patients with untreated acute myelogenous leukemia. When we followed the procedure of Kuhns et al (16), utilizing the low molecular-weight acceptor phenyl-β-D-galactoside, the apparent level of (Fuc→Gal) enzyme activity in our plasma samples was found inversely correlated with the percent of marrow blast cells, as reported by these investigators. The rationale for this difference is currently under study. With normal plasmas, the rate of incorporation of fucose from a GDP-fucose donor into fucosyl-phenylgalactoside proceeds at about 7% of the rate found with desialized fetuin. High molecular-weight fucose acceptors in leukemic plasma might more effectively compete with a low versus a high molecular-weight fucose exogenous acceptor. Alternatively, two (Fuc→Gal) enzymes may be present in plasma, differing in their preference for a fucose acceptor. The major plasma fucosyltransferases apparently prefer high molecular-weight acceptors (11). Bauer et al (2) found an elevation of *both* (Fuc→Gal and Fuc→GlcNac) fucosyltransferases in plasma of patients with neoplastic disease, but did not distinguish between endogenous acceptor activity of the two enzymes.

Bauer et al (1) also reported a 5–20 nmols/g level of GDP-fucose in liver and hepatoma tissue. Since liver is a site of synthesis of this nucleotide sugar (17), such a finding is not unexpected. But, the presence of endogenous GDP-fucose in plasma could alter the significance of our results. Detectable levels of nucleotide sugars have never been reported in plasma, perhaps because of the presence of degradative enzymes. In the present study, the exogenous level of radioactive GDP-fucose was slightly above the K_m for the (Fuc→Gal) enzyme, and about 50% of the K_m for the (Fuc→GlcNac) enzyme (K_m values from refs 14 and 18). The presence of significant levels of endogenous GDP-fucose in leukemic plasmas would mean that we *underestimate* the true fucosyltransferase level in such plasmas. Since the K_m for the (Fuc→Gal) enzyme was in the micromolar range (0.05 nmol/50 μl), any endogenous GDP-fucose would need to be less than this order of magnitude for such a K_m to have been derived.

The measurement of these two enzymes in plasma can provide useful information on the marrow status of patients with acute myelogenous leukemias, and may serve as a supplement for frequent marrow determinations. Furthermore, the level of the GDP-fucose:*N*-acetylglucosaminide (Fuc→GlcNac) fucosyltransferase affords an index of regeneration of normal marrow function following myelosuppressive drug therapy.

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Phase II Study of ICRF-159 Versus Combination *cis*-Dichlorodiammineplatinum(II) and DTIC in Patients With Disseminated Malignant Melanoma [1,2]

Brief Reports and Preliminary Communications

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Disseminated malignant melanoma is a neoplasm that is most refractory to available cytotoxic chemotherapeutic agents. The rather rapid clinical course from the onset of systemic chemotherapy until death in observed patients attests to its aggressive clinical behavior, with survival times reported in the range of 16–18 weeks (1). Most consistent chemotherapeutic responses have been obtained utilizing DTIC (2) and the nitrosoureas (3). These have been in the 15%–20% response range. The quantity and quality of responses prompt further efforts at augmenting proven antitumor effects of available agents as well as a search for new active agents.

cis-Dichlorodiammineplatinum(II) (*cis*-platinum) has been shown to be therapeutically potentiating in the L1210 leukemia system with a number of antitumor agents including a carboxamide (4), but specifically has not demonstrated potentiation with a nitrosourea, BCNU. *cis*-Platinum is one of a group of transitional metal compounds demonstrated to have antineoplastic effects in a number of animal tumor systems (5,6). The spectrum of *cis*-platinum antitumor effect has been well reviewed by Gottlieb and Drewinko (7), with the antitumor response somewhat limited. In general, *cis*-platinum has dose-limiting toxicity primarily affecting the renal system. Other reported toxic effects have involved the gastrointestinal tract, the hematopoietic system, and the eighth cranial nerve.

ICRF-159 has been selected for a number of clinical trials on the basis of its antitumor activity in L1210, Lewis lung, and other model tumor systems (8). Specific cytotoxic action is alleged to occur at the G_2-M phase of cell cycle growth.[4] Toxic effects have included myelosuppression, mainly leukopenia, as well as gastrointestinal toxic effects and alopecia (9,10). ICRF-159 has not previously received adequate clinical trial in untreated patients with disseminated malignant melanoma.

MATERIALS AND METHODS

Thirty-seven patients (26 men and 11 women; mean age, 55 years, median age, 54 years) with disseminated malignant melanoma were included in this study. None of the patients entered into this clinical trial had received previous chemotherapy with any of the study drugs and only two patients had received previous chemotherapy of any type; one had received VP-16-213 and dianhydrogalactitol singly and the other had received a combination of vinblastine, hydroxyurea, and bleomycin. Three of the patients had received previous bacillus Calmette-Guérin as treatment for their disease. Twenty-nine of the 37 patients had visceral dominant disease, most with more than one organ system involved. The eight patients with soft tissue disease were equally distributed in the two treatment groups.

All of the patients were acceptable candidates for cytotoxic chemotherapy with respect to performance score and hematologic status; 33 of the patients were ambulatory outpatients, with only four patients needing bedrest up to 50% of the time. None of the patients were completely bedridden. All patients were randomized into one of the two treatment programs and upon demonstrated failure they received the alternate drug if they were still candidates for study. DTIC was given iv at a dose of 300 mg/m^2 on each of 5 successive days with *cis*-platinum given in this regimen at a dose of 40 mg/m^2 on Day 2 only of each cycle. ICRF-159 was given at a dose of 1000 mg/m^2/day for 3 consecutive days orally.

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Of those patients receiving more than one course of the combination of DTIC and *cis*-platinum, six patients had a decrease in dose of from 10% to 50% while four patients had an increase in dose of 10%. Eight patients receiving ICRF-159 for more than one course had a dose reduction averaging 25%.

All patients were reassessed 4 weeks following completion of each treatment. An objective regression was defined as a decrease of at least 50% of the product of the perpendicular diameters of the indicator lesion(s) lasting a minimum of 4 weeks.

RESULTS AND DISCUSSION

Objective clinical regressions were observed in only three patients, all having received the combination of DTIC and *cis*-platinum. The duration of response was 12 weeks in one patient and response continues at 8 and 9 weeks in the others. Seven patients receiving the combination and eight patients receiving ICRF-159 progressed immediately following the first course of treatment, while 11 patients achieved stability of disease following DTIC and *cis*-platinum and eight patients achieved stability of disease following ICRF-159. The duration of the progression-free interval in the group of patients having stability of disease averaged 13 weeks following DTIC and *cis*-platinum and 13 weeks following ICRF-159 (table 1). One of the ten patients had objective response following crossover therapy with DTIC and *cis*-platinum, with the response continuing at 8 weeks. None of nine patients who received ICRF-159 as secondary treatment experienced an objective regression.

Following primary therapy with ICRF-159, there was a mean survival of 17 weeks with a median of 15 weeks, while following DTIC and *cis*-platinum there was a mean survival of 18 weeks with a median of 12 weeks, both consistent with previous experience. Following secondary therapy, a shortened survival with a mean of 11 and 14 weeks for ICRF-159 and DTIC plus *cis*-platinum, respectively, was observed.

TABLE 1.—Results of primary and secondary treatment

Treatment	No. of regressions/No. of patients entered	
	Visceral dominant disease	Nonvisceral disease
Primary		
DTIC + *cis*-platinum	1/16*	1/4†
ICRF-159	0/13	0/4
Secondary		
DTIC + *cis*-platinum	1/8‡	0/2
ICRF-159	0/9	0/0

*12 wks' duration.
†8+ wks' duration.
‡9+ wks' duration.

TABLE 2.—Toxic effects of primary and secondary treatments

Toxic effect	Treatment	
	DTIC + *cis*-platinum (total: 30 patients)	ICRF-159 (total: 26 patients)
Nausea and vomiting	30	12
Diarrhea	7	5
Alopecia	6	18
Renal	2	0
Wbcs < 3000/mm^3	10*	21†
Platelets < 130,000/mm^3	8	6

*Nadir, 1.8 × 10^3/mm^3; mean duration, 8 days.
†Nadir, 1.7 × 10^3/mm^3; mean duration, 10 days.

The toxicity associated with these programs is displayed in table 2. Renal toxicity was associated in only two instances following DTIC and *cis*-platinum with rather minimal rises in blood creatinine to 1.5 mg/dl, both returning to normal following cessation of this drug. *cis*-Platinum was not given with induced diuresis. No drug deaths were observed in either group although bleeding and sepsis following therapy did occur in one patient who received DTIC and *cis*-platinum.

This combination of DTIC and *cis*-platinum would seem to offer little therapeutic advantage over DTIC alone although the drugs could be given in combination without unexpected problems. The nausea and vomiting associated with this program seemed no more severe than that occurring with DTIC alone. ICRF-159 in previously untreated patients was without clinical effect as defined by an objective diminution in tumor size; thus, this drug would seem to have little place in the management of patients with disseminated malignant melanoma.

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Bleomycin, Methotrexate, and Streptozotocin in Epidermoid Carcinoma of the Lung: An Active Drug Combination With Major Nonhematologic Toxicity [1]

Brief Reports and Preliminary Communications

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The results of combination chemotherapy in patients with epidermoid pulmonary cancer are unimpressive compared to those achieved in small cell carcinoma of the lung. One factor which possibly impairs the effectiveness of multiple-drug treatment in this neoplasm is the limited availability of active nonmyelosuppressive agents. Use of a combination of drugs which included only one with myelosuppressive properties might allow the concomitant administration of full doses of each agent and could lead to improved results.

Bleomycin (BLM), which produces little hematologic toxicity, and methotrexate (MTX) have reported response rates of 13% and 25% respectively in epidermoid lung cancer (1). Given together, they induced regressions in 53% of evaluable patients with epidermoid carcinomas (2). The nitrosoureas CCNU and methyl-CCNU also possess similar single-agent activity in this histologic type of lung tumor (1). At least a single objective response of non-small cell pulmonary carcinoma to the nonmyelosuppressive nitrosourea streptozotocin (STZ) has been observed (3). We therefore studied the effects of combined therapy with BLM, MTX, and STZ in patients with epidermoid carcinoma of the lung.

PATIENTS AND METHODS

Therapy was administered to 16 patients with histologically confirmed, unresectable epidermoid lung cancer. All had measurable or evaluable disease, none had received prior chemotherapy, 15 were men, and the median age was 60 years (range, 49–71). Six had limited disease, defined as previously unirradiated tumor confined to one hemithorax and the ipsilateral supraclavicular area, and ten had extensive disease. Two had had previous pulmonary resection and six (38%) had received radiotherapy (four with curative intent and two to metastatic sites just prior to initiation of chemotherapy). Twelve patients were fully ambulatory and four were confined to bed less than half of the day. Pathologic diagnoses were well or moderately differentiated epidermoid carcinoma in seven patients and poorly differentiated epidermoid carcinoma in nine. Hematologic and renal functions were adequate in all cases as defined by a wbc count $> 4000/\mu l$, a platelet count $> 100{,}000/\mu l$, a serum creatinine < 1.5 mg/dl, and a 24-hour urine protein < 500 mg.

Treatment was planned for 6 weeks in all patients. Thereafter, nonresponding patients with limited disease were referred for radiotherapy, while all patients with extensive disease and responding patients with limited disease were continued on therapy until progressive tumor developed. The BLM dose was 15 units/m² im twice weekly for 6 weeks only, with modification for pulmonary toxicity or severe mucositis. MTX was given at a dose of 25 mg/m² orally twice weekly concurrently with BLM, with dosage reduction for mucositis, myelosuppression, or azotemia. The STZ dose was 1.0 g/m² iv once weekly, with modification for proteinuria or azotemia. Peripheral blood cell counts, serum chemistries, and urine protein determinations were obtained weekly during the first 6 weeks of treatment. In 12 of 13 patients fiberoptic bronchoscopy performed prior to chemotherapy revealed visual and/or pathologic evidence of cancer. Bronchoscopy was repeated at 6 weeks in eight patients.

Partial response was defined as a $\geq 50\%$ decrease in the product of perpendicular diameters of measurable lesions or a substantial decrease in size (confirmed by two investigators) of evaluable lesions. Responses were required to persist for ≥ 1 month without appearance of progressive disease in other sites. All patients were considered evaluable for determination of response rate and survival time.

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TABLE 1.—Sites of disease responding to chemotherapy

Site	No. of responses/ No. of evaluable patients
Lung (evaluated by chest roentgenogram)	4/12
Lung (evaluated by bronchoscopy)	3/8
Peripheral lymph nodes	3/8
Liver (physical examination, scan)	1/1
Esophagus (extrinsic on upper gastrointestinal series)	0/1
Bone (roentgenogram)	0/1

RESULTS

Response and Survival

Ten patients completed 6 weeks of treatment. Four of these continued receiving chemotherapy for a total of 2–6 months. Partial responses were observed in six patients (38%), with a median onset from the start of treatment of 3 weeks and a median duration of 1 month (range, 1–7). Responses of specific sites of disease are given in table 1.

The 16 patients survived a median of 2½ months from the beginning of therapy (range, 1–11+ months). Median survival of responding patients was 3 months, not markedly better than the 2-month median survival of nonresponding patients. The six patients with limited disease (who included one of the six partial responders) lived a median of 4 months, 2 months more than the ten patients with extensive disease. All six patients who lived > 4 months were fully ambulatory at the start of treatment.

Toxicity

During the first 6 weeks of therapy, means of 70%, 60%, and 83% of the projected doses of BLM, MTX, and STZ respectively were actually given to our patients. Dosage reductions were most often due to mucositis and proteinuria. Table 2 details the maximum toxic effects observed in each patient. Nonhematologic toxicity (vomiting, mucositis, and weight loss) was moderate to severe in the majority of patients and led to refusal to continue therapy in three. The severity of weight loss was no different in responding and nonresponding patients. In contrast, hematologic side effects were not observed or were mild in all but three patients. The two patients (13%) with severe myelosuppression, however, died of toxicity. Abnormalities of renal function were not detected in these two patients. While pulmonary toxicity was never noted, mild azotemia (BUN < 30 mg/100 ml) was seen in one patient.

DISCUSSION

Several drug combinations have recently been reported to yield high response rates in non-small cell lung cancer, but the superiority of combination chemotherapy to single-agent treatment in these diseases is not often confirmed in randomized trials (4). The objective response rate of 38% which we observed in our study of epidermoid carcinoma of the lung is similar to the more optimistic results achieved in recent phase II studies of other drug combinations (4). However, the low therapeutic index of our chemotherapy limited its effectiveness in prolonging survival and illustrates why a high

TABLE 2.—Maximum toxic effects of chemotherapy in 16 patients*

Toxicity	No. of patients			
	None	Mild	Moderate	Severe
Wbc count	11	2	1	2
Platelet count	13	1	0	2
Nausea and vomiting	3	1	4	8
Mucositis	6	2	2	6
Hand and finger ulcerations	12	0	3	1
Weight loss during induction	2	3	5	5
Proteinuria	11	2	3	0

*Definitions of toxicities:

	None	Mild	Moderate	Severe
Wbc count (cells/μl)	> 4000	2500–3900	1000–2400	< 1000
Platelet count (cells/μl)	> 100,000	50,000–99,000	20,000–49,000	< 20,000
Mucositis, No. of ulcers	0	1–3	> 3, unable to eat	Confluent
Weight loss (lbs) (not recorded in 1 case)	0–1	2–5	6–10	> 10
Proteinuria, by dipstick	0	1+	2+	3–4+

response rate alone is not necessarily of clinical benefit.

Twice weekly concurrent BLM and MTX produced moderate to severe mucositis in over half of the patients of Lokich and Frei (2), and our results confirm that the toxic effects of these two agents are not wholly nonoverlapping. In a different, more intensive schedule, administration of these two drugs resulted in life-threatening epithelial and hematologic toxicity, albeit with rapid onset of antitumor effects in 71% of the cases with epidermoid cancers (5). Twice weekly BLM and MTX given for 3 weeks in combination with vincristine (6) appeared less toxic and produced a 27% response rate in non-small cell lung cancer. One previously reported drug combination containing BLM, MTX, and a nitrosourea (the drugs utilized in our study) yielded only a 13% response rate in epidermoid carcinoma of the lung without inducing stomatitis (7); however, BLM and MTX were given infrequently and nonconcurrently. Unfortunately, in all of these studies of chemotherapy combinations including BLM and MTX, the frequency of regression of epidermoid cancers appears to correlate with the magnitude of epithelial toxicity, whether treatment was given for ≤ 3 weeks (5,6) or for a longer time period (2,7), as with our patients.

The severe mucositis, vomiting, and weight loss which we noted in our patients are perhaps similar to the "debilitation syndrome" described in the COMB (cyclophosphamide, Oncovin [vincristine], methyl-CCNU, and BLM) regimen (8), which also contains twice weekly BLM and a nitrosourea. Equivalent response rates of 35%–45% in epidermoid lung cancer without "debilitation" were reported by the same authors with a similar combination including BLM given once a week (9). Only if such dose and/or scheduling modifications could preserve the rather high degree of antitumor activity while greatly ameliorating the toxicity would further trials with our chemotherapy regimen appear to be indicated.

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Comparison of Adriamycin With Cyclophosphamide in Patients With Advanced Endometrial Cancer [1]

Brief Reports and Preliminary Communications

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Adenocarcinoma of the uterine corpus is one of the most common gynecologic neoplasms. Control or cure of early, favorable stages of disease is usually achieved by surgery and radiation therapy but a significant number of patients fail treatment or present with more advanced or metastatic disease (1). Hormone therapy is known to produce an approximately 30% incidence of responses in patients with metastasis (2,3), but there are only small series relating to the effectiveness of cytotoxic chemotherapy (4–6). This study was started in 1974 to compare, in patients resistant to progestational agents, the incidence and duration of objective responses induced by adriamycin and by cyclophosphamide. A crossover design was also incorporated to compare these factors in patients already treated by the other drug. These drugs were chosen for study because of their demonstrated activity in other tumor types (7,8) and because there was a possibility that they might subsequently be used in combination.

METHODS

Patients with histologically confirmed carcinoma of the corpus uterus were eligible for study if they had measurable components of local extension or metastasis no longer amenable to standard surgical or radiotherapeutic treatment and if they were expected to survive for ≥ 2 months. They were required to have failed prior progestational therapy and needed to have adequate hepatic, renal, and bone marrow function (bilirubin ≤ 1.5 mg/100 ml, SGOT ≤ 60 IU, BUN ≤ 20 mg/100 ml or creatinine ≤ 1.5 mg/100 ml, wbc count $\geq 5000/mm^3$, and platelet count $\geq 100{,}000/mm^3$). Patients were excluded if they had received prior cytotoxic chemotherapy, had congestive cardiac failure or other severe heart disease, had active infection, had an active second primary tumor, or did not consent to participate.

Pretreatment stratification took into account the extent of disease (pelvis only versus outside pelvis), the Eastern Cooperative Oncology Group performance status (0 or 1 versus 2 or 3), and the histologic grade (well versus moderately or poorly differentiated) of the tumor. Randomization (by a central office) was between two regimens: adriamycin (50 mg/m^2 bolus iv every 3 weeks to a maximum of 550 mg/m^2) or cyclophosphamide (666 mg/m^2 bolus iv every 3 weeks). Treatment was given for ≥ 6 weeks, but if progression occurred or the patient received a maximal dose of adriamycin the patient was eligible to receive the opposite treatment. Conventional provisions for dose reductions due to toxicity were made. Drug doses were escalated by 20% if no toxicity occurred. Regular clinical and laboratory investigations to determine response and toxicity were performed. Tumor response definitions included complete (disappearance of all evidence of disease clinically, radiographically, and by laboratory testing), partial (decrease by $\geq 50\%$ of the sum of the cross-sectional areas of all measured lesions in the absence of progression of any lesion or the appearance of new lesions), no change (change in

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TABLE 1.—Response to chemotherapy

Course	Drug	No. of responses				
		Complete	Partial	No change	Progression	Total
Initial	Adriamycin	1	3	6	11	21
	Cyclophosphamide	0	0	7	12	19
Crossover	Adriamycin	0	1	1	7	9
	Cyclophosphamide	0	1	2	3	6

measurable disease too small to meet the requirements for partial response or progression and no new lesions), and progression (increase in the size of measurable disease by > 25% or the appearance of new lesions). Grades of severity of hematologic toxicity were recorded as none (wbc count ≥ 4500/mm^3, platelet count ≥ 130,000/mm^3), mild-moderate (wbc count 4499–2000/mm^3, platelet count 129,999–50,000/mm^3), severe (wbc count 1999–1000/mm^3, platelet count 49,999–25,000/mm^3), and life threatening (wbc count < 1000/mm^3, platelet count < 25,000/mm^3).

RESULTS

Forty of 47 registered patients were eligible for analysis. Three were excluded because of protocol violations and four because of unavailability of data. Twenty-one patients received adriamycin initially and six subsequently received cyclophosphamide. Of the 19 patients who initially received cyclophosphamide, nine subsequently received adriamycin. Three patients were not evaluable for response because only one dose of the initial drug was received. There was an equal distribution of the stratification parameters between the two treatment groups. The response to the initial treatment is shown in table 1.

There were four responses among 21 patients receiving adriamycin (19%) in comparison with no responses among 19 patients receiving cyclophosphamide. All four patients with responses had a favorable initial performance status and had disease located outside the pelvis. No statistical significance can be attributed to the location of the tumor because all but four of the 37 patients had disease outside the pelvis. Two patients had well-differentiated and two patients had moderately or poorly differentiated tumors. The responses occurred in the cervical lymph nodes (two patients), the abdomen (one patient), and the lung and pelvis (one patient). The responses occurred between 21 and 200 days after the start of treatment and lasted from 21+ to 524 days.

The response to the "crossover" treatments is also summarized in table 1.

The data from the induction and crossover steps on toxicity have been merged, providing 29 patients evaluable for adriamycin toxicity and 23 for cyclophosphamide toxicity. Adriamycin produced 15 instances of mild-moderate, three instances of severe, and two instances of life-threatening hematologic toxicities. The figures for cyclophosphamide were 12 instances of mild-moderate, two instances of severe, and one instance of life-threatening hematologic toxicities. Severe gastrointestinal toxicity occurred in three patients on each drug. No overt cardiac toxicity occurred but there was one instance of severe cystitis with cyclophosphamide.

The data on survival demonstrate no effect due to tumor response or the type of chemotherapy. The initial performance status, however, was an excellent discriminant. The median survival time for patients who were ambulatory (performance status 0 or 1) was 280 days with nine of 21 patients still alive and the median survival time for patients with a poor performance status (2 or 3) was 92 days with three of 19 patients still alive.

DISCUSSION

These results indicate that adriamycin is active in this disease. Using the method of Jeffries (9) and making the assumption that the probabilities of a response on initial and crossover steps are equal and independent, the chance that the apparent superiority of adriamycin over cyclophosphamide is real is 13:1. The sample size is too small to evaluate cross resistance. The 20% response incidence to adriamycin is lower than the 17 responses among 44 patients reported by the Gynecologic Oncology Group (10). This may be related to the higher dose (60 mg/m^2 iv every 3 weeks) that was used in their study which produced "moderate but significant myelosuppression." The drug doses chosen in this study were purposely conservative because of the anticipated frequent combination of advanced age and prior pelvic irradiation in the treated patients.

This policy was probably justified since eight severe or life-threatening toxicities to adriamycin and six similar toxicities to cyclophosphamide occurred. Different criteria for eligibility will possibly allow more intensive chemotherapy.

The lack of influence of tumor shrinkage or of choice of chemotherapy on survival is difficult to explain except that the powerful effect of the initial performance status may have had a masking effect.

This study points out that single-agent chemotherapy, using these drugs, has limited application for advanced or metastatic endometrial cancer resistant to progestational agents. Preliminary studies of several combinations have been reported and these leads are being currently followed by several cooperative clinical groups.

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Mitomycin C in Advanced Adenocarcinoma and Large Cell Carcinoma of the Lung [1,2]

Brief Reports and Preliminary Communications

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Mitomycin C is an antitumor antibiotic isolated from *Streptomyces caespitosus*. Initial reports from clinical trials in Japan were encouraging with an overall response rate of 37%, documented in a review by Frank and Osterberg (1). Attempts to reproduce the Japanese data following a daily dose schedule resulted in brief responses with serious hematopoietic toxic effects (2–4).

The studies of Sokoloff et al in mice with Ehrlich ascites tumors demonstrated increased therapeutic effectiveness with an intermittent treatment schedule when compared to a daily administration of mitomycin C (5,6). Phase I and II clinical studies utilizing a single-dose intermittent dosage schedule have demonstrated therapeutic antitumor activity (7–9).

There has been little success with single-agent and combination chemotherapy in advanced adenocarcinoma and large cell carcinoma of the lung. Responses to mitomycin C in these tumors have been reported, but the number of patients treated is small and meaningful interpretation is difficult (10,11). Thus, it appeared reasonable to initiate a clinical trial of mitomycin C, utilizing the single-dose intermittent schedule, against these tumor types.

MATERIALS AND METHODS

This study was restricted to patients who had histologically proven incurable adenocarcinoma or large cell carcinoma of the lung. All slides were submitted to a referral pathologist for confirmation of diagnosis. Other criteria for eligibility included the following: written consent as to the investigational nature of the study, a life expectancy of at least 8 weeks, no prior chemotherapy with mitomycin C, measurable disease outside a concurrently or previously irradiated site, initial wbc count > 4000/mm^3 and platelet count >100,000/mm^3, adequate renal function as defined by a serum creatinine ≤1.5 mg/100 ml and BUN ≤ 25 mg/100 ml, and no chemotherapy given in the previous 3 weeks.

Mitomycin C was given at an initial dose of 20 mg/m^2 by iv push into an established iv line and repeated in 6 weeks. Third and subsequent courses of mitomycin C were reduced to 10 mg/m^2. Each treatment course was administered provided there was sufficient evidence of recovery from the nadir leukocyte and platelet counts observed with the previous course of therapy. Patients were evaluated while receiving therapy with serial cbc counts, chest roentgenograms, liver and renal function tests, and tumor measurements. A 4-week survival while receiving therapy constituted an adequate trial.

Complete remission (CR) was defined as the disappearance of all clinical evidence of active tumor for a minimum of 4 weeks together with a disappearance of all tumor-related symptoms. Partial remission (PR) was defined as > 50% decrease in the sum of the products of the largest perpendicular diameters of all measured lesions lasting for at least 4 weeks in the absence of significant increase in any one lesion or the appearance of new lesions. Progressive disease was defined as unequivocal increase of any measured lesion or the appearance of new lesions.

Demographic Data

Of the 37 patients entered into the study one died 5 days after initiation of treatment and was considered an early death while another patient received concurrent radiation therapy to the measurable tumor mass and was clearly a protocol violation. Of the remaining 35 evaluable patients

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TABLE 1.—Hematologic toxicity

Course (dose)	No. of patients	Wbc nadir (× 10^3 cells/mm^3)		Median days to— Nadir	Median days to— Recovery*	No. of patients	Platelet nadir (× 10^3 cells/mm^3)		Median days to— Nadir	Median days to— Recovery†
1 (20 mg/m^2)	32	Mean	4.5	14.5	23	29‡	Mean	153	14	25
		Median	4.2				Median	120		
		Range	0.3–9.8	12–20	20–27		Range	14–430	5–27	12–41
2 (20 mg/m^2)	16	Mean	4.7	16	25	14§	Mean	140	18	30
		Median	4.9				Median	106		
		Range	2.1–7.7	10–28	13–37		Range	13–365	14–25	20–43
3 (10 mg/m^2)	4	Mean	6.4			4	Mean	122		
		Median	6.8				Median	125		
		Range	3.6–8.6				Range	53–185		
4 (10 mg/m^2)	2	Mean	3.0			2	Mean	94		
		Median	3.0				Median	94		
		Range	1.7–4.4				Range	20–167		

* > 4000 cells/mm^3.
† > 100,000 platelets/mm^3.
‡ Nadir counts not available for 3 patients.
§ Nadir counts not available for 2 patients.

(26 men, nine women) the median age was 56 years (range, 38–77 years), and the median Karnofsky performance status was 65 (range, 40–90). Two patients had received prior chemotherapy (5-fluorouracil [5-FU]) and had clearly progressive disease at the time of entrance into this study.

RESULTS

Response

Of the 20 patients in the adenocarcinoma group there was one CR and four PRs for an overall response rate of 25%. In the large cell group (15 patients) two PRs were seen for a response rate of 13%. Median duration of remission for both responder groups combined was 12 weeks (range, 4–32 weeks). All responses were noted in the first course of treatment.

Toxicity

Nonhematologic toxicity could be evaluated in all 35 patients and consisted of nausea (3%), vomiting (6%), and diarrhea (6%). One patient developed alveolar fibrosis that may be attributed to mitomycin C since other etiologies were eliminated at postmortem examination. No renal toxicity was observed.

The hematologic toxicity is outlined in table 1. As can be seen there was a tendency for cumulative myelosuppression (course 1 versus course 2, course 3 versus course 4) although there was no delay in administering second or subsequent courses of therapy because of failure to recover from bone marrow suppression. Analysis of all courses of treatment demonstrated that 43% of patients had a platelet count nadir <100,000/mm^3 while only 20% had leukocyte count nadirs < 3000/mm^3.

DISCUSSION

Based on both animal data and our clinical experience with mitomycin C utilizing a single-dose intermittent schedule of drug administration, we explored the clinical usefulness of this agent against advanced, incurable adenocarcinoma and large cell carcinoma of the lung. While the overall response rates observed (25% for adenocarcinoma and 13% for large cell carcinoma) were not particularly high, it appears that the drug does demonstrate antitumor activity equivalent to other compounds tested.

All responses occurred during the first course of therapy and were of a relatively brief duration, which suggests that the drug's effectiveness may be enhanced when combined with other agents that have shown clinical activity. Indeed, a recent report (12) indicates that when mitomycin C was combined with 5-FU and adriamycin for advanced adenocarcinoma of the lung, a higher overall response rate was observed (36%) and a longer duration of response was noted (6.5+ months) when compared to our study of mitomycin C alone. Obviously, both studies contain a relatively small number of patients and a prospective randomized study is indicated to determine the efficacy of combination versus single-agent therapy.

We found thrombocytopenia and leukopenia to be significant, especially with succeeding courses of

therapy. A dosage reduction is imperative if mitomycin C is to be utilized in combination with other myelosuppressive agents.

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Chemotherapy for Benign and Locally Aggressive Tumors: Report of Two Cases [1]

Brief Reports and Preliminary Communications

Michael F. Hensley, Ayten Cangir,* Jan van Eys, and Helmuth Goepfert [2]

Some locally aggressive tumors in children may be invasive and difficult to treat. They can infiltrate surrounding tissues, making complete surgical removal impossible. In such cases, local recurrences after surgery are common, and further surgical resection of the tumor may not be accomplished without a significant functional and/or aesthetic compromise. Most importantly, some benign tumors, such as angiofibromas of the nasopharynx, are potentially life-threatening because of their tendency to bleed.

The use of chemotherapeutic agents in the treatment of invasive and recurrent benign tumors has been limited to hormonal trials (1). With the exception of a recent trial (2), the effectiveness of other antineoplastic agents in the treatment of these tumors has not been tested. This paper presents two patients who had clinically aggressive tumors that regressed dramatically after chemotherapy with a combination of adriamycin (ADR) and DTIC.

CASE REPORTS

Patient 1

A 15-year-old white boy had a profuse epistaxis following a bicycle accident in July 1973. The epistaxis subsided in several days but recurred the following month. The patient was hospitalized and the bleeding was controlled by nasal packing. After 2 days, the patient was discharged; he returned in December 1973 with recurrent nasal bleeding. Because of recurrent epistaxis, a carotid arteriogram was performed; it demonstrated a large tumor of the nasopharynx. On December 13, 1973, ligation of the left external carotid artery was done with transatrial, transoral, and transpalatal resection of a juvenile nasopharyngeal fibroma. The patient did well until April 1974 when the tumor in the nasopharynx and epistaxis recurred. Further resection was done and radioactive gold seed implants were placed in the nasopharynx to deliver 4000 rads of radiation. Abnormal roentgenographic films were obtained in August 1974, and the patient was referred to the M. D. Anderson Hospital and Tumor Institute.

Examination of the patient indicated a large nasopharyngeal mass extending into the left side of the nose with destruction of the septum. Cerebral arteriograms showed the presence of a mass involving the nasopharynx, nasal passage, pterygoid fossa, ethmoid sphenoid sinuses, and left maxillary antrum. Because of the size and the location of the tumor, past therapy, and the potential for hemorrhage, chemotherapy was considered to be the preferred treatment. Combination chemotherapy with ADR and DTIC was started in October 1974 on the following dosage schedule: ADR, 60 mg/m^2 on Day 1, and DTIC, 250 mg/m^2 on Days 1–5. Chemotherapeutic courses were repeated at 3-week intervals. Improvement of symptoms occurred after the first course of chemotherapy; the patient noted that he could breathe in and out through his nose, something that nasal passage obstruction by the tumor had prevented prior to therapy. In January 1975, after four courses of chemotherapy, tomograms of the paranasal sinuses showed a $> 50\%$ reduction in tumor size. The maximum permissible cumulative dose (500 mg/m^2) of ADR was reached by the end of March 1975. A repeat arteriogram, obtained because of possible tumor recurrence, showed complete disappearance of the tumor. Chemotherapy was discontinued. The patient returned on June 28, 1976, with recurrent nasal bleeding. Sinus tumor films and carotid arteriograms demonstrated questionable tumor recurrence in the left side of the nasopharynx. On September 11, 1976, a mass was found in the nasopharynx and the patient had surgery to obtain multiple nasopharyngeal specimens. Results of the biopsy indicated that this mass was a pyogenic granuloma. The patient was referred to an otolaryngologist for nasopharyngeal toilet and further management of the pyogenic granuloma.

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Patient 2

A 3 1/2-year-old white girl was in good health until December 2, 1975, when a slight swelling of the left side of the mandible was noticed by her mother. Her local physician treated the swelling with antibiotics. She returned on December 15 with progressive swelling of the face. Roentgenograms revealed a large lytic lesion of the left side of the mandible. Tissue obtained by curettage was diagnosed as fibroma. During the next 2 months, the mass rapidly grew in size. The patient was referred to the M. D. Anderson Hospital and Tumor Institute.

On physical examination there was a large, soft tissue mass in the left side of the mandibular area. The mass partially broke through the molar area on the left. The tumor measured >7 cm at its greatest diameter and was externally palpable 1 cm from the zygoma superiorly and inferiorly extended into the neck. The facial nerve was not involved, but the tumor extended to the anterior tonsillar pillars and bulged into the area of the pterygoids resulting in partial blockage of the oropharynx. Movement of the mandible was limited and the child could not fully open her mouth. Repeat roentgenograms showed a large destructive lesion involving the angle and the ascending ramus of the left side of the mandible. According to the head and neck surgeon consulted, removal of the mass would require a disfiguring hemimandibulectomy. Chemotherapy, with the objective of reducing tumor size to facilitate surgical resection, was started. Because of the excellent response of patient 1 to combination chemotherapy with ADR and DTIC, patient 2 received the same combination with the same dose schedule. After the second course of chemotherapy there was a marked diminution in the size of the tumor. Treatment courses were continued and all visible tumor disappeared. ADR-induced myocarditis developed after a cumulative dose of 420 mg/m^2 had been administered; this was successfully treated with digitalis and diuretics. Surgical resection of residual tumor was performed on September 21, 1976.

DISCUSSION

Although surgery is the primary treatment for most benign tumors, some tumors may not be resectable because of location, infiltration of surrounding tissues, or both. Radiation therapy has occasionally been successful in the management of aggressive benign tumors (3). However, radiation may fail to control tumor growth, as in patient 1. Nevertheless, most would continue to treat unresectable angiofibroma with local radiation to avoid side effects of systemic chemotherapy although late effects of radiation are not negligible (4–6).

The selection of ADR and DTIC for management of patient 1 was based on good response rates obtained with this combination in a number of miscellaneous childhood tumors (7). The excellent response obtained for patient 1 was the basis for selecting the same treatment regimen for patient 2.

The potential therapeutic effects of anticancer drugs have not been tested in histologically benign but clinically aggressive and life-threatening tumors. The use of antineoplastic drugs for the management of nonmalignant tumors has been controversial and considered unethical because of the unknown long-term effects of these drugs. Nevertheless, the use of all possible methods of treatment cannot be disputed when a patient's life becomes endangered or a vital function becomes disrupted by the growing tumor.

In summary, the observation of significant tumor regression as a result of using the combination of ADR and DTIC in one patient with angiofibroma of the nasopharynx and in another patient with a desmoid fibrosarcoma of the jaw is encouraging for the introduction of antineoplastic drugs in the treatment of surgically unresectable invasive benign tumors.

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Laetrile Toxicity: A Report of Two Patients [1]

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Brief Reports and Preliminary Communications

Laetrile, a cyanogenic glycoside found in seeds of apricots, peaches, and bitter almonds, is an unproven remedy for cancer (1) that has received recent widespread attention in the lay press. The use of Laetrile has been legalized in several states even though antineoplastic activity has not been demonstrated for a broad range of predictive animal tumor systems (2–4), and there is no clear evidence of clinical effectiveness in man.

One of the strongest arguments for approval voiced by the proponents of Laetrile has been the assertion that this material is void of the usual toxic effects associated with conventional cancer therapy. Both the public and medical profession have accepted this claim of safety on faith and it has been assumed that Laetrile is, at worst, a nontoxic placebo. However, experiments in rats have shown that lethal cyanide toxicity can be produced with Laetrile administration, especially by the oral route and when given in conjunction with β-glycosidase. It has been calculated that a lethal dose of cyanide could be obtained from an oral dose of 25 mg/kg in man (5). There are, however, no published reports of systematic toxicologic evaluations of patients receiving Laetrile.

We report here on two patients with toxic reactions that occurred in association with Laetrile administration and we challenge the present claims of its safety.

Patient 1

This 48-year-old white woman was diagnosed as having a well-differentiated lymphoproliferative disorder in 1965, at which time she was treated with cobalt radiation. For the past 8 years she has been treated with prednisone, 20 mg/day, and intermittently has received various forms of unconventional therapy, but not Laetrile. In July 1976, the patient was referred to the Division of Medical Oncology at Georgetown University Hospital after she first evidenced progression of disease with marked hepatosplenomegaly. Following an initial evaluation, it was recommended that she begin a course of chlorambucil but she failed to return for followup.

In January 1977 she returned after having spent 3 months in a nutritional clinic in Germany where she received Wobe-Mugos and pyrogenic iv medication but not Laetrile. She was found to have widespread lymphadenopathy, a liver span of 17 cm, and a spleen which was palpable 12 cm below the left costal margin. Her hemoglobin level was 8.5 g/dl, her wbc count was 72,000/mm³ with 80% well-differentiated lymphocytes, and her platelet count was 63,000/mm³. Bone marrow aspirates were unsuccessful, and bone biopsies demonstrated complete replacement with well-differentiated lymphocytes. She was treated with chlorambucil, 0.12 mg/kg for 7 days, and prednisone was continued. The wbc count improved to 15,000/mm³ while her hemoglobin level and platelet count were not appreciably changed. The lymphadenopathy and hepatosplenomegaly did not change significantly. She again failed to appear for followup.

On April 25, 1977, she returned with complaints of malaise, nausea, backache, headache, abdominal cramps, and fever without chills. She was found to be febrile to 38.8°C (102°F) with a diffuse, nonpruritic, erythematous macular rash which was most pronounced over the trunk but spared the face. An abdominal examination demonstrated diffuse tenderness, but bowel sounds were audible and there were no signs of peritoneal inflammation. The lymphadenopathy had not changed, the liver span was 18 cm, and the spleen remained palpable 11 cm below the left costal margin. Her hemoglobin level was 8 g/dl, her wbc count was 7000/mm³ with 89% lymphocytes, 6% polymorphonuclear cells, and 5% bands, and her platelet count was 69,000/mm³. The patient had spent 3 weeks in Mexico (from February 23 through mid-March) receiving iv Laetrile (Amygdalina, Cytopharma de Mexico, reg. No. 84954SSA), 6 g/day. She continued receiving iv Laetrile, 6 g/week, in addition to oral Laetrile, 500 mg three times a day, following her return to the United

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States. She had not taken chlorambucil since January but had continued with prednisone.

In view of her relative neutropenia and fever, she was hospitalized. Blood, urine, stool, and throat cultures were taken and she was treated symptomatically with iv fluids. Laetrile was discontinued, but daily oral prednisone was maintained. All cultures proved negative for pathogenic bacterial growth, and within 24 hours after admission her temperature was normal, and her rash and abdominal cramps rapidly resolved within 2 days. She signed herself out against medical advice.

On June 5, she resumed Laetrile against our advice and again received 6 g/week iv and 500 mg three times a day orally. She was readmitted to the hospital on June 20 with headache, nausea, abdominal cramps, rash, and fever to 38°C (102°F), the exact syndrome leading to her admission on April 25. Again, cultures were negative for pathogenic bacterial growth, the blood cyanide level was 1 μg/dl, and a skin biopsy was consistent with a drug eruption with perivascular infiltration with lymphocytes. Laetrile was discontinued on admission and the fever, rash, and abdominal discomfort cleared within 48 hours.

Patient 2

In June 1976 this 46-year-old man was found to have large cell anaplastic carcinoma of the lung metastatic to the left parietotemporal region of the cerebrum. He was treated with radiation therapy, 3000 rads, to the whole brain and to the mediastinum followed by chemotherapy with chlorozotocin, a phase I investigational drug. In September 1976 he experienced a generalized major motor seizure and was given Dilantin (diphenylhydantoin). Neurologic examination at that time demonstrated an isolated left medial rectus paresis, with moderate dilation and reduced light reflex of the left eye, normal skeletal muscle strength, normal deep tendon reflexes, but a positive snout reflex.

He was admitted to the hospital in March 1977 with a 1-week history of progressive neuromuscular symptoms. On examination there was bilateral lid ptosis, complete on the left and 75% on the right. Bilateral medial rectus paresis was present, but the other cranial nerves were intact. He had bilateral upper and lower extremity proximal muscle weakness to three fifths of expected strength with normoactive reflexes, a right Babinski reflex, and a snout reflex. The blood glucose was 91 mg/dl, and serum electrolyte, calcium, and CPK levels were normal. It was learned from the patient that he had been taking a 500-mg Laetrile tablet daily since September 1976 in addition to the prescribed Dilantin. Laetrile was discontinued, and within 48 hours his ptosis and proximal muscle weakness improved dramatically and resolved completely in 6 days. Computerized axial tomogram of the brain showed a new lesion in the tectum and he was given Decadron (dexamethasone) and additional CNS radiation.

The patient has not taken Laetrile and has continued to receive chlorozotocin, and on followup visits, he has not had recurrence of either ptosis or proximal muscle weakness.

DISCUSSION

The initial rationale for the use of Laetrile as an anticancer agent was based on the assumption that the compound would be taken up by cancer cells and then hydrolyzed by β-glucosidase to liberate cyanide. It was subsequently demonstrated that animal tissues have only trace β-glycosidase activity and tumor cells even less (6).

Although there has been no systematic evaluation of Laetrile's potential for toxicity at "therapeutic" doses in man, reference has been made in the German literature to vertigo, nausea, and tachycardia (suggestive of cyanide poisoning), and hemoglobinuria in patients receiving Laetrile and β-glucosidase (Nieper HA, as quoted in ref 5, p 1359). Cyanide poisoning and death have been reported in young children ingesting apricot and other fruit seeds containing cyanogenic glycosides (7,8). Sixty bitter almonds, containing approximately 1.8 g of amygdalin, constitute a lethal dose for adults (9). Acute cyanide poisoning is manifest by giddiness, hyperpnea, headache, palpitation, cyanosis, unconsciousness, and death. A chronic neurologic toxicity has been described in natives ingesting cassava which contains cyanogenic glycosides. At the present time it is not possible to determine whether the toxic effects observed in our patients were directly related to cyanide, an intermediate metabolite of amygdalin, or perhaps an adulterant in the specific batch of compound.

Our first patient presented with a rash, gastrointestinal symptoms, and fever requiring hospitalization; these symptoms and signs improved dramatically after discontinuation of Laetrile. However, within 3 weeks after resumption of this material these symptoms recurred. This patient had a form of malignancy readily recognized to be responsive to conventional chemotherapy, but nevertheless she sought an ineffective and toxic treatment.

Our second patient had a potentially more serious reaction. This patient presented with ptosis and weakness of the extremities that cleared rapidly after stopping Laetrile. Although the location of the

metastasis might have contributed to the ptosis, this abnormality cleared before either radiotherapy or Decadron was initiated. It is probable that the reduction in muscular strength was mediated either by direct toxicity to muscle or by interference with neuromuscular transmission. Unfortunately, we did not have the opportunity to perform the appropriate neuromuscular tests to elucidate the exact mechanism.

We feel that these case reports represent definite toxic effects resulting from the administration of Laetrile. The syndromes could have been misdiagnosed as a manifestation of disease in patients with malignant diseases. The measures taken to insure quality control of Laetrile and the concentration of "active agent" as well as that of potentially toxic contaminants are not known. It cannot be assumed that Laetrile is nontoxic or that this compound has not already contributed to the death of patients with malignant diseases. Therefore, it behooves the advocates making claims of the effectiveness of Laetrile not only to provide data which demonstrate objective benefits from this material, but also to furnish detailed toxicologic information which is regularly required for any experimental therapy.

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Phase II Study of Hexamethylmelamine in Women With Advanced Breast Cancer Refractory to Standard Cytotoxic Therapy [1,2,3]

Brief Reports and Preliminary Communications

John M. Denefrio and Charles L. Vogel [4,5,*]

Hexamethylmelamine (HMM) has been reported to be effective as a single agent in 12%–32% (1–3) of patients with metastatic breast carcinoma. These data however, were derived from broad phase II studies including many tumor types and in which most patients had not been previously treated with alkylating agents (2,3). HMM has also been used in combination with vincristine and methotrexate, and a preliminary report documented that five of 11 (45%) patients with advanced breast cancer responded to treatment (4). While HMM is thought to act primarily by alkylation, there are in vitro studies that suggest that this might not be the only mode of action (5). These data are supported by reports that patients with cervical carcinoma refractory to standard alkylating agents have had tumor regression with HMM (6), and patients failing to respond to HMM have responded to melphalan (7). The need for a disease-specific phase II study of HMM in breast cancer coupled with HMM's possible efficacy in alkylating agent-resistant neoplasms led to this study in patients with breast cancer refractory to standard cytotoxic treatment.

METHODS

Nineteen women (mean age, 47 years; median, 52 years) with histologically proven, advanced breast cancer were entered into the trial. The mean duration of metastatic disease prior to HMM therapy was 24 months (range, 6–54 months). All patients had at least one measurable lesion and all had previously been treated with alkylating agents and other cytotoxic drugs (*see* table 1). No patient had received chemotherapy or hormonal therapy within 4 weeks and only one patient received concurrent CNS radiation.

Pretreatment evaluation included: complete history and physical examination; tumor measurements; performance status; complete blood cell and platelet counts; urinalysis; serum urea nitrogen, creatinine, alkaline phosphatase, GOT, bilirubin, uric acid, total protein, albumin, calcium, phosphorus, and lactate dehydrogenase; electrocardiogram; chest roentgenogram; liver scan; bone scan; and bone roentgenograms of scan-positive areas. Complete blood cell and platelet counts were done weekly while tumor measurements, chest roentgenogram, and serum chemistries were repeated every 3 weeks. Every 6 weeks bone roentgenograms were repeated to follow lytic bone lesions. The definition

TABLE 1.—Characteristics of 19 patients entered into the study

Characteristic	No. of patients (%)
Menopausal status (at time of first recurrence)	
Premenopausal	9(47)
Postmenopausal	10(53)
Dominant lesion	
Soft tissue	8(42)
Visceral	7(37)
Bone	4(21)
No. of metastatic sites	
1 or 2	13(68)
≥ 3	6(32)
Prior hormonal treatment	17(89)
Prior chemotherapy with—*	
C + F	19(100)
C + A + F	18(94)
C + A + F + M	17(89)
C + A + F + M + V	6(32)

*C = cyclophosphamide; F = 5-FU; A = adriamycin; M = methotrexate; V = vincristine.

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[2]This pilot study was conducted for the Southeastern Cancer Study Group and was supported in part by Public Health Service grant CA-05641 from the National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare, and in part by a grant from the American Cancer Society (Dr. Denefrio).

[3]Hexamethylmelamine was supplied by the Cancer Therapy Evaluation Program, Division of Cancer Treatment (DCT), NCI.

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[5]We appreciate the cooperation of Drs. Howard Lessner, Michael Troner, Lawrence Broder, Charles Hinkes, and Daniel D. Nixon for entering patients into this study.

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of an objective response was standard and in conformity with recently published guidelines of Hayward et al (8). The pretreatment absolute granulocyte count was $> 1500/mm^3$ and the platelet count was $> 100,000/mm^3$ in all patients. The Karnofsky performance status was $\geq 60\%$ in all patients and $\geq 70\%$ in 18 of the 19 patients.

HMM was administered orally in four divided doses (after meals and at bedtime) at a dose of 320 $mg/m^2/day \times 21$ days, followed by a 21–day rest period before repeating the cycle. This dose and schedule of HMM were those recommended by the DCT, NCI, at the time of activation of the protocol (July 1976). There was no concurrent antineoplastic treatment given except for CNS radiation in one patient. No pyridoxine was given at any time during the study.

RESULTS

Of the 19 patients entered into the study, 15 were evaluable for response. Two patients were not evaluable because of death from CNS metastases prior to completion of one 6-week cycle. One of these two patients had no clinical signs of CNS disease prior to entry into the study while the other was receiving CNS radiation for documented metastatic disease. Two patients were taken off the study early because of severe neurotoxicity requiring discontinuation of HMM after receiving $< 50\%$ of the calculated dose. Of the evaluable patients, three of 15 completed two complete cycles while 12 of 15 completed only one cycle. None of these patients had an objective response although one had stable disease for one 6-week cycle but refused further treatment because of moderately severe gastrointestinal toxicity.

Toxicity was primarily gastrointestinal and neurologic but there were no drug-related deaths or permanent sequelae. Seven of 19 patients developed moderately severe nausea and vomiting only partially relieved by antiemetics. Three of these seven required de-escalation of the dose and in two of these three patients therapy was stopped because of intolerable nausea and vomiting.

Neurologic toxicity occurred in ten of 19 patients but was mild in seven of ten patients. Mild toxicity was depression or anxiety which correlated in time with the treatment course and was more severe than that frequently associated with advanced malignant disease. Three of 19 patients experienced moderate to severe neurologic toxicity. One of these three patients developed a lower extremity tremor which resolved rapidly after the HMM treatment course ended. Two patients developed a hallucinatory and confusional state requiring discontinuation of HMM after administration of $< 50\%$ of the expected dose which rendered them unevaluable for antitumor response.

There was no hematologic toxicity resulting from HMM. One patient had a platelet count of 38,000/mm^3 but had been extensively treated with mitomycin C until 4 weeks prior to HMM (platelet count, $100,000/mm^3$ and decreasing at start of treatment).

At the time of this report, the mean survival for the 15 patients evaluable for response is 6.0+ months (range, 1.5–13+ months) after initiation of HMM, with nine of 15 patients still alive. Of the survivors, six of nine have had progressive disease despite other therapies while three of nine patients have responded to other treatment. One patient has had stabilization of previously progressive disease for 2+ months while receiving vincristine and mitomycin C. Two patients have achieved a partial remission, one with methotrexate for 6.0+ months and one with aminoglutethimide for 5.0+ months, with both patients still in remission at the time of this report.

DISCUSSION

There is an obvious need for effective treatment in breast cancer refractory to standard chemotherapy including cyclophosphamide, vincristine, methotrexate, 5-fluorouracil (5-FU), and adriamycin. Despite reports suggesting activity for HMM in breast cancer patients (1–3) and efficacy in the treatment of cervical cancer refractory to alkylating agents (6), we found no response to HMM in 15 breast cancer patients refractory to standard cytotoxic drugs. The lack of response occurred despite treating half of the patients to moderately severe gastrointestinal or neurologic toxicity. Ahmann has commented on difficulties in interpreting published results of phase II studies in breast cancer and has emphasized careful analysis of pretreatment characteristics of the population being treated (9). Our patients had received extensive prior treatment and had had metastatic disease for a mean of 24.0 months. The disease-free interval prior to development of metastatic disease was 21 months (range, 0–168 months). Most patients (18 of 19) had a Karnofsky performance status of $\geq 70\%$; 68% (13 of 19) had only one or two areas of metastatic disease; 42% (eight of 19) had primarily soft tissue disease, and only 37% (seven of 19) had visceral disease with two of seven being parenchymal lung nodules; the average survival was 6.0+ months after initiation of treatment with three of nine survivors responding to alternative treatment. These data indicate that while these patients had advanced disease they were an ade-

quate population to provide a valid test of the efficacy of HMM.

CONCLUSION

In the present dose and schedule, we found HMM to be ineffective in the management of metastatic breast cancer already refractory to standard chemotherapy and hormonal therapeutic approaches.

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LETTERS

Development of Hodgkin's Disease in a Patient Receiving Procarbazine, CCNU, and Vincristine Therapy for a Gemistocytic Astrocytoma[1]

We wish to report the case of a patient who developed Hodgkin's disease following radiation therapy and multiple courses of chemotherapy for treatment of a gemistocytic astrocytoma over a 2½-year period.

A 57-year-old woman developed confusion, dysphasia, and upper-extremity weakness in November 1972. On December 12, 1972, she had subtotal resection of a left frontal gemistocytic astrocytoma, and for 1½ months thereafter she received whole-brain radiation therapy (5100 rads). Two months after this therapy, she developed increasing dysphasia and right hemiparesis. Twelve weeks after radiation therapy was completed, chemotherapy with procarbazine, CCNU, and vincristine (PCV No. 1) was initiated.

She received 32 courses of PCV No. 1 at 4-week intervals. During the first two courses, procarbazine (100 mg/m² orally every hour she was asleep) was administered for 14 days beginning on Day 1, CCNU (75 mg/m² iv) was administered on Day 1, and vincristine (1.4 mg/m² iv) was administered on Days 1 and 8. Vincristine was reduced to 50% dosage on the sixth course, 40% on the 11th–30th courses, and was discontinued thereafter because of neuropathy with orthostatic hypotension. Because of myelosuppression, CCNU was reduced 50% on the third to 32nd courses, and procarbazine was reduced 50% on the third to tenth and 15th–32nd courses, and 25% on the 11th–14th courses. Over the 32-month period, the patient received 1.28 g of CCNU and 21 g of procarbazine.

Throughout treatment, the patient had slight dysphasia and mild right hemiparesis. She was able to undertake moderate activities, but she had little energy and appeared frail. Her radionuclide scans, after showing initial improvement, remained stable. Computer tomograms remained stable with a small left frontal area of contrast enhancement adjacent to an area of low density.

Four weeks after her 32nd course of PCV No. 1, she became weaker with decreased mentation and she developed fever, nausea, vomiting, and diarrhea. Although a retroperitoneal tumor was suspected 6 weeks prior to her death, no biopsy was performed because of her poor physical condition.

Sections of the tumor biopsied in 1972 had revealed sheets of plump, moderately pleomorphic gemistocytic astrocytes with eccentric nuclei that displayed rare mitotic figures, as well as occasional nests of fibrillary astrocytes with occasional bizarre nuclei. There was no vascular proliferation or necrosis.

At autopsy, the brain at the surgical site contained a smooth-walled simple cyst with groups of gemistocytic and fibrillary astrocytes at the cortical surface bordering the cyst wall; they showed little pleomorphism and no mitoses. The major portion of the cyst wall contained scattered, well-differentiated astrocytes and hemosiderin granules. Multiple sections revealed no spread of tumor away from the cyst wall.

Necrotic nodules were found in the lungs, liver, adrenals, and spleen at autopsy. The enlarged perihilar and retroperitoneal lymph nodes contained necrotic centers, which were diffusely infiltrated by bizarre histiocytes, Reed-Sternberg cells, scattered eosinophils, and lymphocytes. Central necrosis with a surrounding fibroblastic response was apparent, most markedly within the lungs. The findings were consistent with lymphocyte-depleted Hodgkin's disease.

It has become increasingly apparent that oncolytic agents applied in a course to suppress an initial neoplasm can contribute to the formation of a second malignant tumor (1), which is generated either by reaction with DNA, leading to a direct chemical carcinogenic mutation, or by suppressing the natural immune mechanism. Although vincristine and CCNU have not yet been implicated as major carcinogenic agents, procarbazine has been shown to have major carcinogenic properties (2) and may have contributed to the development of the second neoplasm in this case. There is a high incidence of neoplastic disease associated with iatrogenic as well as naturally occurring immunologic deficits.

In Penn's series of 160 patients who showed 166 cases of second malignancies after treatment with

[1]Supported by Public Health Service grant CA-13525 from the National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare, and American Cancer Society Faculty Research Award FRA-155 (Dr. Levin).

chemotherapy, only three patients developed Hodgkin's disease as the second malignant tumor (3). The Hodgkin's disease in our patient appeared well within the temporal range cited for these cases. Although our patient was treated with a different combination of carcinogens than the patients in Penn's group, her primary tumor was just as resistant to treatment as those in Penn's group, for Hodgkin's disease developed during treatment with three drugs that should have been therapeutically effective.

It is not possible to ascertain what initiated the development of Hodgkin's disease in this patient because multiple factors could have contributed to neoplastic growth. It is possible that with longer exposure to treatment with oncolytic chemotherapy, more patients with primary brain tumors will develop second neoplasms.

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Symmetrical Mental Neuropathy in Breast Cancer

The "numb chin syndrome" is reportedly a grave prognostic sign in patients with diverse malignant neoplasms (1–4). Most patients have unilateral paresthesias usually from metastatic involvement of the calvarium (2). This report describes a patient with symmetrical mental neuropathy as the initial manifestation of disseminated breast cancer.

A 56-year-old woman had a left modified radical mastectomy in March 1975 for adenocarcinoma with metastases to four of 13 axillary lymph nodes. There was no radiation therapy or chemotherapy administered following mastectomy. In September 1976 she noticed severe paresthesias of the chin but no evidence of recurrent cancer. Multiple left chest wall nodules later appeared but regressed with diethylstilbestrol. By March 1977 the mandibular symptoms worsened and there was a recurrence of the chest wall lesion which was biopsy-proven adenocarcinoma.

Pertinent physical findings included the symmetrical and bilateral absence of a pin-and-touch sensation on the chin and diffuse nodularity on the left chest wall. Skeletal roentgenograms, including skull and mandibular views, were negative. A bone scan showed diffusely increased uptake in the frontal bones. Intra-oral roentgenograms revealed loss of trabecular patterns and multiple osteolytic defects in the mandible contiguous with the central and lateral incisors and the cuspids.

Breast cancer comprised only 0.3% of all malignant lesions of the mandible and maxilla in a series of 594 patients (5). However, the mandibular metastases in our patient were the first indication of systemic malignant disease. This case report demonstrates the usefulness of intra-oral or panoramic dental radiograms in clarifying the etiology of mandibular symptoms in patients with cancer.

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High-Dose Methotrexate, Not a Conventional Treatment

High-dose methotrexate (MTX) has become a popular mode of cancer treatment, especially for osteogenic sarcoma (1). In the last annual report to the Food and Drug Administration on high-dose MTX (1977) the Investigational Drug Branch of the Na-

tional Cancer Institute (NCI) listed 110 studies using this therapy, with the majority (46%) of protocols for osteogenic sarcoma. The total quantity of MTX supplied by the NCI last year approached 100 kg and its value was approximately one million dollars. In the face of the magnitude of this program it is essential that the clinical advantage of high-dose regimens over more "conventional" dose regimens of MTX be shown.

MTX toxicity is certainly reversed by citrovorum factor (CF), and in animal studies, Goldin et al showed that administration of CF increases the margin of safety for MTX and that high doses with greater effectiveness could be administered with less lethal toxicity (2). However, convincing evidence for increased effectiveness of high-dose MTX over "low-dose" MTX is scanty or lacking in human studies. Only a few direct comparisons between high and low doses of MTX have been done. Djerassi and colleagues in their pioneer work on high-dose MTX indicated that some patients "had apparently been resistant to more conventional doses and schedules of administration of MTX prior to entering this (high-dose MTX) program" (3) and the response to the high-dose MTX in those patients may point to the increased effectiveness of a high-dose MTX-CF regimen. No comparison of two groups of leukemia patients, one receiving conventional-dose and the other high-dose MTX-CF, was ever done.

An apparent advantageous response rate in osteogenic sarcoma has been reported with high-dose MTX-CF (4). However, the experience, except in an adjuvant setting, is by no means extensive. The response of osteogenic sarcoma to MTX in conventional doses was never studied, and curiously, in soft tissue sarcoma responses have been obtained with low-dose MTX (5) although high-dose MTX-CF regimens have not been active. In the adjuvant setting the favorable experience of high-dose MTX-CF has not been universally shown.

Only three randomized studies of high-dose MTX-CF versus conventional doses have been reported. The earliest study by Levitt et al (6) reported an apparent improved therapeutic index for high-dose MTX-CF in head and neck cancer. This study compared MTX given at maximally tolerated doses as 30-hour iv infusions (starting at 80 mg/m^2 and escalating until toxicity was observed) given every 2 weeks against high-dose MTX (240 mg/m^2 with escalations to 1080 mg/m^2) given as 36–42-hour iv infusions with CF rescue every 2 weeks. Two more recent studies, one conducted by the Southeastern Cancer Study Group (SECSG) and the other by the Eastern Cooperative Oncology Group (ECOG), did not confirm this superiority. The first (7), reported by Vogler and Jacobs for the SECSG, compared three MTX regimens: 60 mg/m^2 iv weekly, versus 15 mg/m^2 orally every 6 hours × four doses weekly, versus 125 mg/m^2 orally every 6 hours × four doses + CF rescue (5 mg orally × 6) weekly in breast cancer, colon carcinoma, and head and neck tumors. In this study there was no difference in antitumor activity among the three regimens. The second study (8) was by the ECOG and compared MTX, 40 mg/m^2 iv weekly, against biweekly high-dose MTX, 240 mg/m^2 iv followed at 42 hours by CF, 25 mg every 6 hours × eight doses, versus a combination regimen using biweekly high-dose MTX + CF with cyclophosphamide (500 mg/m^2 iv) and cytosine arabinoside (300 mg/m^2 iv). Patients with advanced epidermoid carcinoma of the head and neck were entered and 243 cases were eventually randomized. A 24% overall response rate was obtained without significant differences being observed in response among the three protocol arms. Drug-related mortality was approximately 5%. There were survival differences among the responders in the three arms which clearly favored the conventional-dose weekly MTX arm! The "high doses" in these studies were relatively low when compared to the current high-dose MTX schedules; however, they still suggest that an increase in MTX dose does not necessarily increase its therapeutic activity.

The present generation of studies using high-dose MTX-CF is highly variable with regard to the maximum dose of MTX, the rapidity of dose escalation, the duration of the infusion, the timing and dose of CF rescue, and the interval between courses. It is hoped that the influence of these variables on toxicity and efficacy will be subject to intensive, continued study.

In the meantime, the recent report of 6% mortality attributed to high-dose MTX (9) belies the belief that this schedule is usually associated with little toxicity. Currently used high-dose MTX regimens cannot be considered at present the optimal treatment for any condition without additional confirmatory evidence. Comparative trials with other rescue regimens and against the conventional-dose regimens should be carried out to establish such an unequivocal superiority for high-dose MTX. Only evidence of this kind will justify the risks and high expense of this treatment modality.

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Acute Cyclophosphamide Hypersensitivity Reaction: Possible Lack of Cross-Sensitivity to Mechlorethamine and Isophosphamide

Ross and Chabner (1) recently reported the case of a patient with hypersensitivity to topical mechlorethamine who subsequently developed acute allergic reactions to iv mechlorethamine and cyclophosphamide. Their report cautioned against the use of cyclophosphamide in patients with mechlorethamine allergy. Although extreme caution is justified in this regard, our experience with a patient with cyclophosphamide allergy suggests a lack of uniformity in cross-sensitivity between various alkylating agents.

The patient in reference is a 61-year-old white woman who was found to have small cell carcinoma of the left lung in January 1977. She was treated by her private oncologist with a combination of radiotherapy to the primary tumor and chemotherapy with cyclophosphamide given in a dose of 300 mg/m^2/day iv × 4 days every 3 weeks. She tolerated the first two courses of cyclophosphamide well; however, following the first dose of the third course she experienced generalized urticaria resulting in immediate discontinuation of the drug. The skin reaction was persistent and required systemic corticosteroids before it resolved. Her chemotherapy was then changed to mechlorethamine and the first dose of 20 mg was given iv in March 1977 and was tolerated without any clinical allergic manifestations. The patient was subsequently transferred to our care and to date has received four courses of isophosphamide as part of a combination regimen for small cell carcinoma with no recurrence of urticaria or evidence of other allergic reactions.

Cyclophosphamide is a commonly used antineoplastic and immunosuppressive agent rarely noted to cause acute or delayed hypersensitivity reactions. However, the reports of Ross and Chabner (1), Lakin and Cahill (2), and Karchmer and Hansen (3) along with our case report document the possibility of acute urticarial and/or anaphylactic reactions occurring after cyclophosphamide. All patients had such reactions after prior repeated exposure to cyclophosphamide or, as in the case of Ross and Chabner, after topical mechlorethamine, a closely related alkylating agent. It has been suggested that these reactions are type 1 hypersensitivity reactions mediated by IgE-type antibodies (3). However, the specific antigenic stimulus is unknown and may be unchanged drug or one of its metabolites.

Based on their case, Ross and Chabner suggested that hypersensitivity reactions to alkylating agents may require both chloroethyl alkylating moieties attached to the same nitrogen atom. After apparent allergy to cyclophosphamide, our patient and Karchmer and Hansen's patient did subsequently tolerate, without reaction, mechlorethamine and chlorambucil, respectively (alkylating agents with both chloroethyl moieties on the same nitrogen atom). Our patient also tolerated isophosphamide without reaction, an alkylating agent differing in chemical structure from cyclophosphamide solely by the chloroethyl groups being on different nitrogen atoms. It appears then that a hypersensitivity reaction to one alkylating agent usually does not imply cross-sensitivity with other alkylating agents. Since the antigenic stimulus and the mechanism involved in these reactions are not yet quite clear, one must exercise caution in substituting one alkylating agent for another in patients with documented acute allergic reactions to this class of drugs.

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ANNOUNCEMENTS

International Symposium on the CNS Complications of Malignant Disease, 1978

Southhampton, England, April 16–19, 1978

The International Symposium on the CNS Complications of Malignant Disease will be held April 16–19, 1978, at the Medical and Biological Sciences Building, Southampton, England.

Each day of the Scientific Program will be devoted to two main topics: April 17, diagnosis and assessment and direct and indirect clinical consequences of CNS infiltration; April 18, clinical management and specific problems of therapy; April 19, iatrogenic complications of therapy and new and experimental approaches to the investigation, management, and treatment of CNS disease.

All sessions will include reviews by invited speakers and papers selected from submitted abstracts. Each participant is invited to submit an abstract of a paper related to the topics listed in the program. The closing date for receipt of abstracts of the symposium is ***February 14, 1978***. The official language of the symposium is English.

The registration fees are 40 pounds for participating delegates and 15 pounds for accompanying persons. Early registration is advised. ***For registration and abstract submission forms and further information on this symposium contact***: Mr. A. Hayes, Symposium Secretariat, Wessex Oncology Service, Royal South Hants Hospital, Southampton 509 4PE, England.

Seminars in Oncology at Roswell Park Memorial Institute

Buffalo, New York

There will be seminars on "New Leads in Cancer Therapeutics" (March 3, 1978) and "Cancer of the Lung" (March 9, 1978) at the Roswell Park Memorial Institute, organized by the Departments of Experimental Therapeutics and Thoracic Surgery, respectively. The chairpersons are Drs. Enrico Mihich and Ronald Vincent, respectively.

There is a $10 registration fee for each seminar. ***For further information contact:*** Ms. Claudia Lee, Cancer Control, Roswell Park Memorial Institute, 666 Elm St, Buffalo, NY 14263.

Third Annual "Symposium on Advances in Cancer Treatment Research"

Baltimore, Maryland, May 18–20, 1978

The Baltimore Cancer Research Center, The National Cancer Institute, and the Program of Continuing Education of the University of Maryland School of Medicine are pleased to announce their Third Annual "Symposium on Advances in Cancer Treatment Research" to be conducted in Baltimore, Maryland, May 18–20, 1978, at the Baltimore Hilton Hotel.

An experienced and internationally recognized faculty will participate. This symposium is designed to update physicians and surgeons in some of the most recent developments in cancer treatment and current research including areas of clinical management, etiology, and cellular biology. Faculty members will be available throughout the symposium to discuss any questions or interests of individual course registrants. A visit to the Baltimore Cancer Research Center can be scheduled for interested persons. This course is accredited for 16 hours of Category 1 credit for the PRA of the AMA by the Program of Continuing Education, University of Maryland School of Medicine.

Symposium on Anticancer Drugs

Chapel Hill, North Carolina, March 30–31, 1978

The Cancer Research Center and the Department of Pharmacology at the University of North Carolina School of Medicine, Chapel Hill, and the Burroughs Wellcome Co, Research Triangle Park, NC, will sponsor this Second Annual Cancer Research Center Symposium on March 30–31, 1978, at the University of North Carolina School of Medicine, Chapel Hill.

Symposium participants will be: Nicholas R. Bachur (National Cancer Institute [NCI], Baltimore, Md), Joseph R. Bertino (Yale University School of Medicine, New Haven, Conn), Vincent H. Bono and Vincent T. Oliverio (NCI, Bethesda, Md), Robert L. Capizzi and Richard V. Wolfenden (University of North Carolina School of Medicine, Chapel Hill, NC), Gertrude B. Elion and George H. Hitchings (Burroughs Wellcome Co, Research Triangle Park, NC), James F. Holland (Mt. Sinai School of Medicine, New York, NY), and Philip S. Schein (Georgetown University School of Medicine, Washington, DC). Additional faculty of the North Carolina School of Medicine, Bowman Gray School of Medicine, and Duke University Medical Center, and scientists from the Burroughs Wellcome Co will participate in the workshops scheduled for the afternoon of March 31st.

The symposium is open and free of charge. *For further information contact:* Ms. Mimi Minkoff, Cancer Research Center, Box 30, Clinical Sciences Bldg, Chapel Hill, NC 27514 or telephone (919) 966-3036.

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